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2017

USP 40 THE UNITED STATES PHARMACOPEIA

NF 35 THE NATIONAL FORMULARY

Volume 1-2

By authority of the United States Pharmacopeial Convention
Prepared by the Council of Experts and its Expert Committees

Official from May 1, 2017

The designation on the cover of this publication, "USP NF 2017," is for ease of identification only. The publication contains two separate compendia: *The United States Pharmacopeia*, Fortieth Revision, and *The National Formulary*, Thirty-Fifth Edition.

THE UNITED STATES PHARMACOPEIAL CONVENTION
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SIX-MONTH IMPLEMENTATION GUIDELINE

The *United States Pharmacopeia–National Formulary* and its supplements become official **six months** after being released to the public. The *USP–NF*, which is released on November 1 of each year, becomes official on May 1 of the following year. This six-month implementation timing gives users more time to bring their methods and procedures into compliance with new and revised *USP–NF* requirements.

The table below describes the official dates of the *USP–NF* and its supplements. The 2016 *USP 39–NF 34*, and its supplements, *Interim Revision Announcements (IRAs)* and *Revision Bulletins* to that edition, will be official until May 1, 2017, at which time the *USP 40–NF 35* becomes official.

Publication	Release Date	Official Date	Official Until
USP 40–NF 35	November 1, 2016	May 1, 2017	May 1, 2018 (except as superseded by supplements, *IRAs*, and *Revision Bulletins*)
First Supplement to the USP 40–NF 35	February 1, 2017	August 1, 2017	May 1, 2018 (except as superseded by *Second Supplement*, *IRAs*, and *Revision Bulletins*)
Second Supplement to the USP 40–NF 35	June 1, 2017	December 1, 2017	May 1, 2018 (except as superseded by *IRAs* and *Revision Bulletins*)
USP 41–NF 36	November 1, 2017	May 1, 2018	May 1, 2019 (except as superseded by supplements, *IRAs*, and *Revision Bulletins*)

The table below gives the details of the *IRAs* that will apply to *USP 40–NF 35*.

IRA	PF Posting Date	Comment Due Date	IRA Posting Date	IRA Official Date
42(1)	January 4, 2017	March 31, 2017	May 27, 2017	July 1, 2017
42(2)	March 1, 2017	May 31, 2017	July 29, 2017	September 1, 2017
42(3)	May 2, 2017	July 31, 2017	September 30, 2017	November 1, 2017
42(4)	July 1, 2017	September 30, 2017	November 25, 2017	January 1, 2018
42(5)	September 1, 2017	November 30, 2017	January 27, 2018	March 1, 2018
42(6)	November 1, 2017	January 31, 2018	March 31, 2018	May 1, 2018

Revision Bulletins published on the USP website become official on the date specified in the *Revision Bulletin*.

NOTICE AND WARNING

Concerning U.S. Patent or Trademark Rights—The inclusion in *The United States Pharmacopeia* or in the *National Formulary* of a monograph on any drug in respect to which patent or trademark rights may exist shall not be deemed, and is not intended as, a grant of, or authority to exercise, any right or privilege protected by such patent or trademark. All such rights and privileges are vested in the patent or trademark owner, and no other person may exercise the same without express permission, authority, or license secured from such patent or trademark owner.

Concerning Use of USP *or* NF *Text*—Attention is called to the fact that *USP* and *NF* text is fully copyrighted. Authors and others wishing to use portions of the text should request permission to do so from the Secretary of the USPC Board of Trustees.

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Contents

VOLUME 1

Mission Statement and Preface vii

People 2015–2020 Revision Cycle xi

- Officers xi
- Board of Trustees xi
- Council of Experts xi
- Expert Committees xii
- In Memoriam xviii

Members of the United States Pharmacopeial Convention, as of May 31, 2016 xix

2015 Recognition of Monograph and Reference Material Donors ... xxvi

Articles of Incorporation xxviii

USP Governance xxix

- Bylaws xxix
- Rules and Procedures xxix
- USP Policies xxix

Admissions xxxiii

- Articles Admitted to *USP 40* by Supplement xxxiii
- New Articles Appearing in *USP 40* That Were Not Included in *USP 39* Including Supplements xxxiv
- Articles Included in *USP 39* But Not Included in *USP 40* xxxv
- Annotated List xxxvi

Notices

- General Notices and Requirements 1

General Chapters

See page 63 for detailed contents

- General Tests and Assays 67
- General Requirements for Tests and Assays 67
- Apparatus for Tests and Assays 107
- Microbiological Tests 111
- Biological Tests and Assays 143
- Chemical Tests and Assays 245
- Physical Tests and Determinations 472
- General Information 829
- Dietary Supplements 2245

Reagents, Indicators, and Solutions 2323

- Reagent Specifications 2324
- Indicators and Indicator Test Papers 2406
- Solutions 2409
  - Buffer Solutions 2409
  - Colorimetric Solutions 2411
  - Test Solutions 2411
  - Volumetric Solutions 2423
- Chromatographic Columns 2436

afroozpharma@yahoo.com

Reference Tables

Containers for Dispensing Capsules and Tablets 2443
Description and Relative Solubility of *USP* and *NF* Articles 2453
Approximate Solubilities of *USP* and *NF* Articles 2513
Atomic Weights 2521
Atomic Masses 2522
Alcoholometric Table 2523
Intrinsic Viscosity Table 2525

Index

Combined Index to *USP 40* and *NF 35* I-1

VOLUME 2

Guide to General Chapters vii

Notices

General Notices and Requirements xi

USP 40

Monographs

Official Monographs for *USP 40*, A–I 2527

Index

Combined Index to *USP 40* and *NF 35* I-1

VOLUME 3

Guide to General Chapters vii

Notices

General Notices and Requirements xi

USP 40

Monographs

Official Monographs for *USP 40*, J–Z 4741

Index

Combined Index to *USP 40* and *NF 35* I-1

VOLUME 4

Guide to General Chapters vii

Notices

General Notices and Requirements xi

Global Health

Official Monographs 6779

Dietary Supplements

Official Monographs 6781

afroozpharma@yahoo.com

NF 35

Admissions

Articles Admitted to *NF 35* by Supplement . . 7483

New Articles Appearing in *NF 35* That Were Not Included in *NF 34* Including Supplements 7483

New Articles Appearing in *NF 35* 7483

Annotated List 7484

Excipients

USP and *NF* Excipients, Listed by Category 7485

Monographs

Official Monographs for *NF 35* 7495

Combined Index to *USP 40* and *NF 35* I-1

afroozpharma@yahoo.com

Mission and Preface *USP 40–NF 35* and *Supplements*

This section provides background information on the United States Pharmacopeial Convention (USP), as well as general information about the 40th revision of the *United States Pharmacopeia* (*USP 40*) and the 35th edition of the *National Formulary* (*NF 35*) and their *Supplements*. Unless otherwise noted, the text in *USP 40–NF 35* is official May 1, 2017, the text in the *First Supplement* to *USP 40–NF 35* is official August 1, 2017, and the text in the *Second Supplement* to *USP 40–NF 35* is official December 1, 2017.

MISSION STATEMENT

USP–NF is published in continuing pursuit of the mission of USP: *To improve global health through public standards and related programs that help ensure the quality, safety, and benefit of medicines and foods.*

HISTORY

USP has a rich history, dating back to 1820, when 11 physicians met in the Senate Chamber of the U.S. Capitol building to establish a pharmacopeia for the United States. Learn more about USP's history and major milestones on the USP website (http://www.usp.org/about-usp/our-history).

CONTENT OF *USP–NF*

USP–NF contains official substance (ingredient) and product monographs for official articles recognized in *USP–NF* (see *General Notices 2.20 Official Articles*). *USP–NF* also includes monographs for compounded preparations. With few exceptions, such as articles covered by *Global Health* monographs, all articles for which monographs are provided in *USP–NF* are legally marketed in the United States or are contained in legally marketed articles. *Global Health* monographs are provided for articles that are not approved or legally marketed in the United States, but that have been approved by a stringent regulatory authority [as defined by the World Health Organization (WHO)] and are used for essential purposes in other parts of the world.

A *USP–NF* monograph for an official substance, product, or preparation may consist of various components, including the article's name; definition; packaging, storage, and other requirements; and a specification. General chapters provide frequently cited procedures, sometimes with acceptance criteria, in order to compile into one location repetitive information that is applicable to many monographs. See *General Notices 3.10 Applicability of Standards* for more information about standards contained in *USP–NF* monographs and general chapters.

New and revised monographs and general chapters and omitted monographs from this edition are indicated in the *Admissions* section.

***USP–NF* Organization**—The *USP–NF* is published online as the *USP–NF Online*. *USP–NF* is also printed as a four-volume set. *Volume 1* includes front matter (*Mission and Preface, People*, governance pages and websites, and *Admissions/Annotations*). It also includes *USP General Notices, General Chapters, Dietary Supplements* general chapters, *Reagents*, and *Reference Tables*. *Volume 2* includes *USP* monographs A–I, and *Volume 3* includes *USP* monographs J–Z. *Volume 4* includes *Global Health* monographs, *Dietary Supplements* monographs, *NF Admissions/Annotations, Excipients*, and *NF* monographs. To facilitate convenient use and reference, all four volumes include the combined index, as well as the *USP General Notices* and the *Guide to General Chapters*. General chapters specific to dietary supplements are included in numerical order with the rest of the general chapters in *USP*. Excipient monographs are usually presented in *NF* but also may appear in *USP* with suitable cross-referencing when they are also drug substances. The *Excipients* section (*Volume 4*) presents a tabulation of excipients by functional category.

Supplements—*Supplements* to *USP–NF* follow a standard schedule each year: the *First Supplement* is published in February and becomes official August 1. The *Second Supplement* is published in June and becomes official December 1. Users of USP print products must retain *Supplements* and check the "Official Text" section of USP's website to have up-to-date official text. The *USP–NF Online* is updated with each *Supplement* or annual revision. Each time a new edition or *Supplement* is released during the subscription period, a new electronic version is issued. The *Index* in each *Supplement* is cumulative and includes citations to the annual revision and, for the *Second Supplement*, citations to the *First Supplement*. The contents of the two *Supplements* are integrated into the annual edition of the following year, along with new official revisions that have been adopted since the *Second Supplement* to the previous compendia.

Revisions to *USP–NF*—*USP–NF* is continuously revised by an exceptional process of public involvement and substantial interaction between USP and its stakeholders, both domestically and internationally. Revisions are presented annually in the *USP–NF* and in twice-yearly *Supplements*, and as *Accelerated Revisions* on USP's website [*Errata, Interim Revision Announcements* (*IRAs*), and *Revision Bulletins*].

Standard Revisions—USP's standard revision process calls for publication of a proposed revision in the *Pharmacopeial Forum (PF)* for a 90-day notice and comment period and, after the revision is approved by the relevant USP Expert Committee, publication in the next *USP–NF* or *Supplement*, as applicable. Learn more about the development and revision process on the USP website (http://www.usp.org/usp-nf/development-process).

Accelerated Revisions—The *Accelerated Revision* process is used to make revisions to *USP–NF* official more quickly than through USP's *Standard Revision* process. Learn more about *Revision Bulletins, Interim Revision Announcements (IRA), Errata*, and the criteria for and implementation of each on the USP website (http://www.usp.org/usp-nf/official-text).

Modification of Compendial References—USP and its Expert Committees periodically deem it necessary to modify general chapter titles or similar text that may be referenced in other standards throughout the *USP–NF*. When this oc-

curs, USP staff undertake a rigorous process for identifying and updating such references. These updates may occur through a routine revision, or, in cases in which an update appears to present no significant change in the affected standard, through a direct update of the reference in that standard without providing an opportunity for notice and comment. In all cases, USP will publish on its website a notice indicating the source change, any resulting references, and whether those references will be updated through a routine revision or a direct update.

Updating Chemical Information—Updates to the Chemical Information section at the beginning of monographs occur on an ongoing basis and are not identified with revision symbols. Chemical names and molecular weights are updated when a monograph undergoes revision to match the official source, *United States Adopted Names* (*USAN*). Chemical structures are updated on a continuous basis.

Chemical names typically reflect the naming conventions at the time of the monograph development or revision. If the nomenclature rules of CAS or IUPAC are significantly changed, the chemical names can be revised or added to implement those rules. Molecular weights are derived from the chemical formula and are based on the table of atomic weights. Atomic weights are recommended by the IUPAC and reflect the isotopic composition of normal terrestrial material. When the IUPAC recommended values are changed, it is understood that the changes in molecular weights will be made in due course.

Graphical representation of the chemical compound structures is intended as a visual aid to help establish chemical identity and is understood to represent one of many possible ways to depict the molecule. Addition of a graphical representation or changes in such representation, that result in the same chemical information, e.g., a flipped chiral molecule or adding a molecule structure, may be introduced outside of the revision process. It is also understood that in the case of tautomerism, the molecule depicted may be one of the tautomers, but it is intended to represent all isomers in equilibrium. Stereogenic centers depicted with plain bonds imply mixtures of pertinent stereomers—enantiomer, diastereomers, epimers (anomers), etc.

Depending on the timing of these updates, users may see a difference in a chemical structure between the publications in *PF* and *USP–NF*, and between the *USP–NF* and The *USP–NF Online*.

Shading and Symbols—Shading is used to identify text that has been modified, added, or deleted since it was last published. Symbols identify the beginning and end of each revision or nonharmonized text. The following table summarizes the types of symbols and the associated subscripts used in USP publications:

Revision Type	Symbol	Subscript
Interim Revision Announcement	●new text●(IRA 1-Jul-2017)	(IRA 1-Jul-2017)*
Revision Bulletin	●new text●(RB 1-Jan-2017)	(RB 1-Jan-2017)*
Text deletion	●●(IRA 1-Jul-2017) or	(IRA 1-Jul-2017)*
	■■1S (USP40) or	1S (USP40)*
	▲▲(USP40)	USP40**
Adopted in *Supplement*	■new text■1S (USP40)	1S or 2S (USP annual edition)*
Adopted in *USP–NF*	▲new text▲ (USP40)	USP annual edition**
Harmonization	◆residual national text or nonharmonized text◆	

* A subscript number or date indicates the *IRA*, *Revision Bulletin*, or *Supplement* in which the revision first appeared.
** An example of a revision that was officially adopted in the *USP–NF* would be ▲USP40.

Revision Type	Symbol	Subscript
Errata	●new text●(ERR 1-Jul-2017)	(ERR 1-Jul-2017)
Chapter references	●new text●(CN 1-May-2017)	(CN 1-May-2017)
	●new text●(Official 1-Dec-2017)	(Official 1-Dec-2017)

* A subscript number or date indicates the *IRA*, *Revision Bulletin*, or *Supplement* in which the revision first appeared.
** An example of a revision that was officially adopted in the *USP–NF* would be ▲USP40.

The following table shows symbols and official dates for *IRAs* and *Supplements* to *USP 40–NF 35*.

IRAs* and *Supplements* to *USP 40–NF 35
Official Dates and Symbols

Supplement	Proposed IRA	Official Date	Symbols
	43(1)	July 1, 2017	●and●(IRA 1-Jul-2017)
1		Aug. 1, 2017	■and■1S (USP40)
	43(2)	Sept. 1, 2017	●and●(IRA 1-Sep-2017)
	43(3)	Nov. 1, 2017	●and●(IRA 1-Nov-2017)
2		Dec. 1, 2017	■and■2S (USP40)
	43(4)	Jan. 1, 2018	●and●(IRA 1-Jan-2018)
	43(5)	Mar. 1, 2018	●and●(IRA 1-Mar-2018)
USP 41–NF 36	43(6)	May 1, 2018	●and●(IRA 1-May-2018)

Also, in the *USP–NF Online*, monographs and general chapters that have been revised but not yet published in the *USP–NF* or its *Supplements* (e.g., as *Accelerated Revisions*) will contain icons that will link to the page on the USP website where the new official text can be viewed. These icons will link to *Accelerated Revisions* (*Revision Bulletins*, *Interim Revision Announcements*, and *Errata*) and *Stage 6 Harmonization* (see *Harmonization Activities* below).

Commentary—For revisions that are published for public review and comment in *PF*, the proposal may advance to official status or be republished in *PF* for further notice and comment. If comments are received, they are considered and incorporated as appropriate by the Expert Committee(s). In cases where proposals advance to official status without republication in *PF*, a summary of comments received and the appropriate Expert Committee's responses are published in the *Commentary* section of the USP website at the time the revision is published.

The *Commentary* is not part of the official text and is not intended to be enforceable by regulatory authorities. Rather, it explains the basis of the Expert Committee's response to public comments. If there is a difference between the contents of the *Commentary* and the official text, the official text prevails. In case of a dispute or question of interpretation, the language of the official text, alone and independent of the *Commentary*, shall prevail.

Print and Electronic Presentations—See *General Notices 2.10 Official Text* for more information about *USP–NF* product formats.

USP–NF Translations—Translations of the *USP–NF* are available in Spanish, Russian, and Chinese. The Spanish translation is current; other translations are based on previous revisions of the *USP–NF*.

USP Reference Standards—The use of USP Reference Standards promotes uniform quality of drugs and supports reliability and consistency by those performing compliance testing and other users of *USP–NF*, including manufacturers, buyers, and regulatory authorities. USP Reference Standards are referenced in specific procedures in both monographs and general chapters. USP advances this material via careful characterization studies and collaborative testing, followed by review and approval of the compendial use of the reference material by Expert Committees of the Council of Experts. The *USP Catalog*, which lists the collection of USP

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Reference Standards, and more information about use and storage, can be accessed on USP's website (http://www.usp.org/reference-standards). This program benefits from the widespread voluntary contribution of suitable materials and test data from pharmaceutical manufacturers.

USP GOVERNING, STANDARDS-SETTING, AND ADVISORY BODIES

USP's governing, standards-setting, and advisory bodies include the USP Convention, the Board of Trustees, the Council of Experts and its Expert Committees, Expert Panels, Subcommittees, Joint Standard-Setting Subcommittees, and staff. Additional volunteer bodies include Stakeholder Forums and Project Teams, which offer stakeholders the opportunity to contribute, through advice and recommendations, to the advancement of USP's standards and processes. Learn about the composition and work of the USP Convention, Board of Trustees, Expert Committees, and Expert Panels on the USP website (http://www.usp.org/about-usp/leadership/governance). Learn more about Stakeholder Forums and Project Teams on the USP website (http://www.usp.org/meetings-courses/stakeholder-forums). A listing of all current Voting Delegates of the USP Convention and members and Government Liaisons to the Council of Experts and its Expert Committees and Expert Panels is included in the *People* section.

Working with the Food and Drug Administration—USP works with the Secretary of the Department of Health and Human Services, and the principal agency in the Department for this work is the Food and Drug Administration (FDA). USP works in many ways with the agency, but the primary interaction is through the Government Liaison Program. The Government Liaison Program allows representatives of FDA and other government agencies to participate in Expert Committee and Expert Panel meetings, enabling interactions between government staff and Expert Committees. Staff in the FDA Centers who are responsible for review of compendial activities provide specific links and opportunities for exchange of comments. The Office of Policy for Pharmaceutical Quality in the Center for Drug Evaluation and Research provides a primary compendial point of contact between FDA and USP.

RULES AND PROCEDURES

Governing Documents—*USP–NF* standards are recognized widely because they are authoritative and science-based and are established by a transparent and credible process. See the *Articles of Incorporation* section in this book; the Bylaws (http://www.usp.org/about-usp/leadership/bylaws), and the *Rules and Procedures of the Council of Experts* (http://www.usp.org/about-usp/leadership/policies-rules). Collectively, these documents serve USP volunteers and staff as the governing principles for USP's standards-setting activities.

LEGAL RECOGNITION

Recognition of *USP–NF*—*USP–NF* is recognized by law and custom in many countries throughout the world. In the United States, the Federal Food, Drug, and Cosmetic Act (FD&C Act) defines the term "official compendium" as the official *USP*, the official *NF*, the official *Homeopathic Pharmacopeia of the United States,* or any supplement to them. *USP–NF* standards play a role in the adulteration and misbranding provisions of the FD&C Act, which apply as well to biologics, a subset of drugs, under the Public Health Service Act (see *General Notices 2.30 Legal Recognition*).

FDA requires that names for articles that are not official must be clearly distinguished and differentiated from any name recognized in an official compendium. Drugs with a name recognized in *USP–NF* also will be considered misbranded unless they meet compendial standards for packaging and labeling (see *General Notices 3.10.10 Applicability of Standards to Drug Products, Drug Substances, and Excipients* for more information).

Drugs—USP's goal is to have substance and drug product monographs in *USP–NF* for FDA-approved drugs in the United States, including chemical and biologic medicines, and their ingredients. USP also provides monographs in *USP–NF* for legally marketed therapeutic products not approved by FDA, e.g., pre-1938 drugs, over-the-counter (OTC) drugs marketed under FDA's OTC Monograph system, dietary supplements, and compounded preparations. The *Global Health* section of *USP–NF* contains monographs for articles that are not approved or legally marketed in the United States, but that have been approved by a stringent regulatory authority (as defined by WHO) and are used for essential purposes in other parts of the world. Conformance with a *USP–NF* monograph, if applicable, is required at all times in the life of an article from production to expiration.

Biologics—In the United States, biologics are considered to be a subset of drugs, whether they are approved by FDA under the FD&C Act [and receive a new drug application (NDA)] or under the Public Health Service Act [PHS Act, where they receive a biologics license application (BLA)]. As a result, all PHS Act biologics are subject to the applicable drug regulatory requirements of the FD&C Act, which means they are required to comply with the adulteration and misbranding provisions of the FD&C Act, including *USP–NF* compendial requirements, to the extent that such requirements apply to a particular biologic product. This is equally so for biologics approved under the longstanding PHS Act "351(a)" pathway, as well as the new "351(k)" pathway for biosimilars added by the 2010 healthcare reform legislation (Biologics Price Competition and Innovation Act, Title VII, Subtitle A of the Patient Protection and Affordable Care Act, Public Law 111-148).

Dietary Supplements—The Dietary Supplement Health and Education Act of 1994 amendments to the FD&C Act provide that a dietary supplement may be deemed a misbranded food if it is covered by the specifications of an official compendium (e.g., *USP–NF*), is represented as conforming to the specifications of an official compendium, and fails to so conform. This contrasts with pharmaceutical products, wherein conformance to applicable compendial standards is mandatory, whether or not the product claims to conform.

Compounded Preparations—Compounding means the preparation, mixing, assembling, altering, packaging, and labeling of a drug or device or other article, as the result of a practitioner's order or in anticipation of such an order based on routine, regularly observed prescribing patterns. USP provides both general chapters and monographs for compounded preparations. Learn more about compounding on the USP website (http://www.usp.org/usp-healthcare-professionals/compounding).

Medical Devices—Section 201(h) of the FD&C Act defines a device as an instrument, apparatus, similar article, or component thereof recognized in *USP–NF*. Section 502(e) of the FD&C Act defines the established name of a device in the absence of an FDA designation of the official name as the official title in an official compendium. Despite these statutory provisions, there is no comparable recognition of USP's role in establishing compendial standards for medical devices as exists for drugs and biologics. Under authority granted by the Food and Drug Administration Modernization Act of 1997, the Center for Devices and Radiological Health recognizes national and international standards, including some *USP* tests and assays, for medical devices.

Nomenclature—For information on the nomenclature development process, the Nomenclature and Labeling Expert Committee, and USP's work with United States

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Adopted Names (USAN), see the USP website (http://www.usp.org/usp-nf/development-process/compendial-nomenclature).

Chemical Names and CAS Registry Numbers—Chemical subtitles given in the monographs are index names used by the Chemical Abstracts Service (CAS) of the American Chemical Society. They are provided only in monographs in which the titles specify substances that are definable chemical entities. The first subtitle is the inverted form of the systematic chemical name developed by CAS for the purpose of the Collective Index (CI). The second subtitle, given in uninverted form, is a preferred IUPAC name (PIN) sanctioned and used by the International Union of Pure and Applied Chemistry (IUPAC). Preferred IUPAC names also are used by the WHO. Occasionally a third subtitle is supplied for historical reasons or when the synonym uses an alternative, but equivalent, naming convention. Monographs with chemical subtitles also generally carry CAS registry numbers. These bracketed numbers function independently of nomenclature as invariant numerical designators of unique, unambiguous chemical substances in the CAS registry, and thus are convenient and widely used.

HARMONIZATION ACTIVITIES

Pharmacopeial Discussion Group—USP harmonizes pharmacopeial excipient monographs and general chapters through the Pharmacopeial Discussion Group (PDG), which includes representatives from the European, Japanese, and United States pharmacopeias, and WHO (as an observer). According to the PDG definition, "a pharmacopeial general chapter or other pharmacopeial document is harmonized when a pharmaceutical substance or product tested by the document's harmonized procedure yields the same results, and the same accept/reject decision is reached." Information regarding PDG, including history, the PDG working procedure, a glossary, and lists of monographs and general chapters that have completed stages 1–6 of the pharmacopeial harmonization process, resulting in an approved USP Stage 6 Harmonization text, is available on USP's website (http://www.usp.org/usp-nf/harmonization).

Other Harmonizing Activities—USP is participating in several harmonizing activities that include all pharmacopeias. These activities, while currently in progress, are working toward Good Pharmacopoeial Practices and optimal public standards for all medicines.

OTHER USP COMPENDIA

USP Compounding Compendium—The *USP Compounding Compendium* is an electronic compendium that includes all compounding-related general chapters from the *USP–NF* as well as the supporting general chapters that are referenced in the compounding general chapters and in *USP–NF General Notices*. The purpose of the *USP Compounding Compendium* is to provide compounding practitioners with convenient access to associated general chapters.

USP Herbal Medicines Compendium—The *USP Herbal Medicines Compendium (HMC)* is an online compendium that will help ensure the quality of the herbal ingredients used in herbal medicines. *HMC* monographs provide quality specifications—tests, procedures, and acceptance criteria—with validated analytical procedures and allied reference materials that aid in conformity assessment. *HMC* can help ingredient manufacturers, herbal product manufacturers, regulatory agencies, and other stakeholders to assess conformance of herbal medicinal ingredients with independent public standards and to control the quality of articles moving in international commerce. The *HMC* is available at https://hmc.usp.org.

USP Dietary Supplements Compendium—The *Dietary Supplements Compendium* combines, in a two-volume set, *USP–NF* standards for dietary supplements, standards and information from the *Food Chemicals Codex*, regulatory and industry documents, and other tools and resources. It is published every two years as a hardcover print edition.

Food Chemicals Codex—The *Food Chemicals Codex* (*FCC*) is a compendium of internationally recognized monograph standards and tests for the purity and quality of food ingredients, e.g., preservatives, flavorings, colorings, and nutrients. *FCC* is published every two years, with supplements every six months, and is available in print and electronic formats. Proposed revisions to *FCC* are available for public viewing and comment through the *FCC Forum*. The *FCC Forum* can be accessed free of charge at forum.foodchemicalscodex.org.

OTHER USP RESOURCES

Chromatographic Columns—This comprehensive reference, previously titled *Chromatographic Reagents*, provides detailed information needed to conduct chromatographic procedures found in *USP–NF*. *Chromatographic Columns* lists the brand names of the column reagents cited in every proposal for new or revised gas- or liquid-chromatographic analytical procedures that have been published in *PF* since 1980. *Chromatographic Columns* also helps to track which column reagents were used to validate analytical procedures that have become official. The branded column reagents list is updated bimonthly and maintained on USP's website.

USP Dictionary—The *USP Dictionary of USAN and International Drug Names* provides, in a single volume, the most up-to-date United States Adopted Names of drugs; official *USP–NF* names; nonproprietary, brand, and chemical names; graphic formulas; molecular formulas and weights; CAS registry numbers and code designations; drug manufacturers; and pharmacologic and therapeutic categories. The *Dictionary* helps to ensure the accuracy of the following: product labeling; reports, articles, and correspondence; FDA regulatory filings; and pharmaceutical package inserts. It is published annually. For more information about the *Dictionary* see the USP website (http://www.usp.org/usp-nf/development-process/compendial-nomenclature).

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People

2015–2020 Revision Cycle

Officers of the USP Convention, Board of Trustees, and the Council of Experts, Expert Committees, and Expert Panels

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University of Arizona College of Medicine, Hillary A. Franke, M.D., M.S.
University of Arkansas for Medical Sciences College of Medicine, Paul L. Prather, Ph.D., B.S.
University of California Davis School of Medicine, Timothy E. Albertson, M.D., M.P.H., Ph.D.
University of California San Francisco School of Medicine, Linda L. Liu, M.D.
University of Chicago Pritzker School of Medicine, Michael L. Maitland, M.D., Ph.D.
University of Cincinnati College of Medicine, Marianne F. Ivey, Pharm.D., M.P.H., FASHP
University of Connecticut Health Center School of Medicine, Kimberly J. Metcalf, Pharm.D.
University of Hawaii John A. Burns School of Medicine, Robert A. Nichols, Ph.D.
University of Illinois College of Medicine at Chicago, Randal A. Skidgel, Ph.D.
University of Iowa Carver College of Medicine, Donald E. Letendre, Pharm.D.
University of Kansas School of Medicine, Sam J. Enna, Ph.D.
University of Kentucky College of Medicine, Lisa A. Cassis, Ph.D.
University of Louisville School of Medicine, Demetra Antimisiaris, Pharm.D., CGP, FASCP
University of Maryland School of Medicine, Margaret M. McCarthy, Ph.D.
University of Massachusetts Medical School, Roy Guharoy, Pharm.D., M.B.A., FCP, FCC, FASHP
University of Miami Miller School of Medicine, Joshua D. Lenchus, D.O., R.Ph., FACP
University of Michigan Medical School, Paul F. Hollenberg, Ph.D.
University of Mississippi School of Medicine, Deborah S. Minor, Pharm.D., FAHA
University of Missouri–Kansas City School of Medicine, James M. Wooten, Pharm.D.
University of Nebraska College of Medicine, L. Charles Murrin, Ph.D.
University of Nevada School of Medicine, Iain L. Buxton, Pharm.D.
University of New Mexico Health Sciences Center School of Medicine, Arti Prasad, M.D.
University of North Dakota School of Medicine and Health Sciences, James E. Porter, Ph.D.
University of Oklahoma College of Medicine, Pramod K. Chetty, M.D.
University of Pennsylvania School of Medicine, Patrick J. Brennan, M.D.
University of Pittsburgh School of Medicine, Dennis P. Swanson, M.S.
University of Puerto Rico School of Medicine, Miguel A. Marrero, M.D.
University of South Alabama College of Medicine, Jack A. DiPalma, B.S., M.D.
University of South Carolina School of Medicine, Kenneth B. Walsh, Ph.D.
University of South Florida Morsani College of Medicine, Shufeng Zhou, M.D., Ph.D.
University of Tennessee, Memphis College of Medicine, Trevor W. Sweatman, Ph.D.
University of Texas Medical School at Houston, Gary C. Rosenfeld, Ph.D.
University of Utah School of Medicine, Richard J. Sperry, M.D., Ph.D.
University of Virginia School of Medicine, Robert J. Meyer, M.D.
University of Washington School of Medicine, Suzanne M. Allen, M.D., M.P.H.
University of Wisconsin School of Medicine and Public Health, F. Michael Hoffmann, Ph.D.
Vanderbilt University School of Medicine, C. Michael Stein, M.D.
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Wake Forest University School of Medicine, Kathy P. Bricker, Pharm.D., BCPS
Washington University School of Medicine in St. Louis, Evan D. Kharasch, M.D., Ph.D.
Wayne State University School of Medicine, Stephen A. Lerner, M.D.
Wright State University Boonshoft School of Medicine, Khalid M. Elased, Ph.D., R.Ph.
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Academic Institutions and Associations Thereof—Colleges and Schools of Pharmacy

Auburn University Harrison School of Pharmacy, William R. Ravis, Ph.D.
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Campbell University School of Pharmacy, Antoine Al-Achi, M.S.Pharm., Ph.D., M.S.
Chicago State University College of Pharmacy, Duc P. Do, Ph.D.
Creighton University School of Pharmacy and Health Professions, J. Christopher Bradberry, Pharm.D.
Drake University College of Pharmacy and Health Sciences, Abebe E. Mengesha, Ph.D.
Duquesne University Mylan School of Pharmacy, Ira S. Buckner, Ph.D.
Ernest Mario School of Pharmacy at Rutgers University, Longqin Hu, Ph.D.
Ferris State University College of Pharmacy, Kim E. Hancock, Ph.D.
Florida A & M University College of Pharmacy and Pharmaceutical Sciences, Mandip Sachdeva, Ph.D.
Hampton University School of Pharmacy, Chengan Du, Ph.D., M.P.H.
Howard University College of Pharmacy, Nursing and Allied Health Sciences, Emmanuel O. Akala, Ph.D.
Idaho State University College of Pharmacy, Kevin W. Cleveland, Pharm.D.
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Southwestern Oklahoma State University School of Pharmacy, Shelly Stockton, Ph.D.
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St. Louis College of Pharmacy, John Pieper, Pharm.D.
SUNY at Buffalo School of Pharmacy and Pharmaceutical Sciences, Alfred T. Reiman, R.Ph.
Temple University School of Pharmacy, Michael Borenstein, Ph.D.
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The University of Arizona College of Pharmacy, Michael Mayersohn, Ph.D.
The University of Iowa College of Pharmacy, Mickey L. Wells, Ph.D.
The University of Louisiana at Monroe College of Pharmacy, Sami M. Nazzal, Ph.D.
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The University of Toledo College of Pharmacy, Kenneth S. Alexander, R.Ph., Ph.D.
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University of Illinois at Chicago College of Pharmacy, Stephanie Y. Crawford, Ph.D., M.P.H.
University of Kansas School of Pharmacy, Christian Schoneich, Ph.D.
University of Kentucky College of Pharmacy, Eric J. Munson, Ph.D.
University of Maryland School of Pharmacy, Natalie D. Eddington, Ph.D.
University of Michigan College of Pharmacy, Gregory E. Amidon, B.S., Ph.D.
University of Minnesota College of Pharmacy, Marilyn K. Speedie, Ph.D.
University of Mississippi School of Pharmacy, Michael A. Repka, D.D.S., Ph.D.
University of Missouri–Kansas City School of Pharmacy, Cydney E. McQueen, Pharm.D.
University of Nebraska Medical Center College of Pharmacy, Michael F. Powell, B.S. Pharm., M.S.
University of New Mexico College of Pharmacy, Lynda S. Welage, B.S., Pharm.D.
University of Oklahoma College of Pharmacy, Vibhudutta Awasthi, Ph.D., M.Pharm.
University of Pittsburgh School of Pharmacy, Michael A. Zemaitis, Ph.D.
University of Rhode Island College of Pharmacy, David R. Worthen, Ph.D., J.D.
University of São Paulo College of Pharmacy, Prof. Terezinha de Jesus Andreoli Pinto
University of Southern California School of Pharmacy, Stan G. Louie, Pharm.D.
University of Tennessee Health Science Center College of Pharmacy, Laura A. Thoma, Pharm.D., B.S.
University of the Pacific Thomas J. Long School of Pharmacy and Health Sciences, Xiaoling Li, Ph.D.
University of the Sciences in Philadelphia, Philadelphia College of Pharmacy, Daniel A. Hussar, Ph.D.
University of Utah College of Pharmacy, John W. Mauger, Ph.D.
University of Washington School of Pharmacy, Thomas K. Hazlet, Pharm.D., Dr.P.H.
University of Wisconsin–Madison School of Pharmacy, James E. DeMuth, Ph.D., R.Ph.
University of Wyoming School of Pharmacy, Kurt Dolence, Ph.D.
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West Virginia University School of Pharmacy, Arthur I. Jacknowitz, Pharm.D.
Western University of Health Sciences College of Pharmacy, Sunil Prabhu, Pharm.D., R.Ph.
Wilkes University Nesbitt School of Pharmacy, Harvey A. Jacobs, Ph.D.
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American Diabetes Association, Jane L. Chiang, M.D.
American Health Quality Association, Kenneth Mishler, R.Ph., Pharm.D.
American Heart Association, Robert Lee Page II, Pharm.D., M.S.P.H., FCCP, FAHA, FASHP, FASCP, BCPS, CGP
Arthritis Foundation, John H. Klippel, M.D.
Center for Science in the Public Interest, David G. Schardt, M.S.
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ECRI Institute, Jeffrey C. Lerner, Ph.D.
Institute for Safe Medication Practices, Michael R. Cohen, R.Ph., M.S., Sc.D., FASHP
National Consumers League, Karin Bolte, J.D.
National Council on Patient Information and Education, William R. Bullman, M.A.M.
National Osteoporosis Foundation, Amy Porter
William J. Clinton Foundation, Rodger W. Stringham, Ph.D.

Governmental Bodies or Divisions or Associations Thereof

Agency for Healthcare Research and Quality, Scott R. Smith, Ph.D.
Argentine Pharmacopoeia, Héctor A. Giuliani, Pharm.D.
Association of Food and Drug Officials, Cynthia T. Culmo
Brazilian Pharmacopoeia Commission, Mônica da Luz Carvalho Soares
Brazil's National Institute of Quality Control in Health, Filipe Soares Quirino Silva, Ph.D.
British Pharmacopoeia Commission, James Pound
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Centers for Medicare & Medicaid Services, Jeffrey A. Kelman, M.D.
China National Center for Food Safety Risk Assessment, Junshi Chen, M.D.
Chinese Pharmacopoeia Commission, Zhang Wei
Department of Veterans Affairs Veterans Health Administration, Timothy J. Stroup, R.Ph.
European Directorate for the Quality of Medicines and HealthCare, Susanne Keitel
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FDA Center for Devices and Radiological Health, Marilyn M. Lightfoote, M.D., Ph.D.
FDA Center for Drug Evaluation and Research, Pallavi Nithyanandan, Ph.D.
FDA Center for Food Safety and Applied Nutrition, Daniel E. Folmer, Ph.D.
FDA Center for Veterinary Medicine, Janis R. Messenheimer, D.V.M.
Federal Commission for Protection Against Sanitary Risks, Rocio del Carmen Alatorre Eden-Wynter, M.C.
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Food and Drugs Authority, Ghana, Eric Karikari-Boateng, M.S.
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General Directorate of Medicines, Supplies and Drugs, Laura Cerón, Pharm.D.
Health Canada, Karen Reynolds
Health Canada, Food Directorate, Barbara Lee
Health Canada, Natural and Non-prescription Health Products Directorate, Jeannine R. Ritchot
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Indonesian Pharmacopoeia Commission, Augustine Zaini, M.S.
International Trade Administration—U.S. Department of Commerce, James D. Rice, B.A., M.A.
Japan's Ministry of Health Labour and Welfare, Pharmaceuticals and Medical Devices Agency, Nobuo Uemura
Jordan Food and Drug Administration, Dr. Lubna R. Qusous
Ministry of Food and Drug Safety, Dr. Jin-Ho Wang
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Ministry of Health of Turkey, Filiz Bütev Koç, Ph.D.
Morocco's Directorate of Medicines and Pharmacy, Laila Hakkou
National Agency for Food and Drug Administration and Control, Dr. Monica H. Eimunjeze
National Association of Boards of Pharmacy, Elizabeth Scott Russell, B.S.Pharm.
National Center for Quality Control, Ofelia del Rosario Villalva Rojas, Q.F.
National Centre for Expertise of Drugs, Medical Products and Medical Equipment, Ardak U. Tulegenova, Ph.D.
National Drug Authority of Uganda, Gordon Katende Sematiko
National Health Surveillance Agency, Lais Santana Dantas, B.S.
National Institute for Biological Standards and Control, Adrian F. Bristow, Ph.D.
National Institute of Drug and Food Surveillance, Eduardo Vergel Bayona
National Institute of Metrology, Quality and Technology, Valnei Smarçaro da Cunha, Ph.D.
National Institute of Standards and Technology, Michael J. Tarlov, Ph.D.
National Institutes for Food and Drug Control, Bo Li, Ph.D.
National Institutes of Health, Robert DeChristoforo, R.Ph., M.S., FASHP
Permanent Commission of the Pharmacopoeia of the United Mexican States, Maria del Carmen Becerril-Martinez
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Philippine Pharmacopeia, Maria Lourdes C. Santiago, B.S.Pharm., M.S.Pharm.
Saudi Food and Drug Authority, Abdullah S. Alhomoud, M.S.
State Administration of Ukraine on Medicinal Products, Andrii V. Shovkovyi
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Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines, Oleksandr I. Gryzodub, Ph.D.
United States Agency for International Development, Anthony F. Boni
United States Air Force–Office of the Surgeon General, Deborah Myers, R.Ph., M.B.A.
United States Army–Office of the Surgeon General, John Spain, Pharm.D., BCPS
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United States Navy Bureau of Medicine and Surgery–Office of the Surgeon General, Thinh V. Ha, Pharm.D., M.B.A., M.S.
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American Academy of Neurology, Jack J. Chen, Pharm.D., FASCP, FCCP
American Academy of Nurse Practitioners, Jan Towers, Ph.D., NP-C
American Academy of Ophthalmology, Wiley A. Chambers, M.D.
American Academy of Pediatrics, Hank Farrar, M.D.
American Academy of Physician Assistants, Marie-Michele Leger, M.P.H., PA-C
American Academy of Veterinary Pharmacology and Therapeutics, Carol A. Davis, Ph.D.
American Association of Pharmaceutical Scientists, Christopher McCurdy, Ph.D., R.Ph.
American Botanical Council, Mark Blumenthal
American Chemical Society, Denise L. Creech
American College of Clinical Pharmacology, Peter Wiernik, M.D.
American College of Clinical Pharmacy, C. Edwin Webb, Pharm.D., M.P.H.
American College of Physicians, Brian L. Strom, M.D.
American Dental Association, Eugenio D. Beltrán-Aguilar, D.M.D., M.P.H., M.S., Dr.P.H.
American Dental Education Association, Vahn A. Lewis, Pharm.D., Ph.D.
American Geriatrics Society, Todd P. Semla, M.S., Pharm.D., BCPS, FCCP, AGSF
American Medical Association, Barry D. Dickinson, Ph.D.
American Nurses Association, Rita Munley Gallagher, Ph.D., R.N.
American Optometric Association, Jimmy D. Bartlett, O.D.
American Pharmacists Association, Thomas E. Menighan, R.Ph., M.B.A., FAPhA
American Public Health Association, Deborah K. Walker, Ed.D.
American Society for Clinical Pharmacology and Therapeutics, Patricia W. Slattum, Pharm.D., Ph.D.
American Society for Microbiology, Amy L. Leber, Ph.D.
American Society for Nutrition, Robert M. Russell, M.D.
American Society for Parenteral and Enteral Nutrition, Gordon S. Sacks, Pharm.D., BCNSP, FCCP
American Society for Pharmacology and Experimental Therapeutics, Mary Paine, Ph.D.
American Society for Quality, Donald C. Singer, M.S.
American Society of Anesthesiologists, H.A. Tillmann Hein, M.D., Ph.D.
American Society of Consultant Pharmacists, Arnold E. Clayman, Ph.D., FASHP
American Society of Health-System Pharmacists, Paul W. Abramowitz, Pharm.D., FASHP
American Veterinary Medical Association, Donald C. Sawyer, D.V.M., Ph.D.
Argentine Association of Industrial Pharmacy and Biochemistry, Federico Montes de Oca, M.B.A.
Brazilian National Association of Compounding Pharmacists, Maria do Carmo Garcez
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Canadian Pharmacists Association, Perry Eisenschmid, M.B.A.
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European Federation for Pharmaceutical Sciences, Hendrik J. de Jong, Ph.D.
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International Academy of Compounding Pharmacists, John Voliva, R.Ph.
International Council of Nurses, David C. Benton, RGN, RMN, BSC, MPhil, FFNF, FRCN
International Society for Cellular Therapy, Joseph C. Laning, Ph.D.
International Society for Pharmaceutical Engineering, Stephen M. Tyler, M.S.
National Alliance of State Pharmacy Associations, Rebecca P. Snead, R.Ph., B.S.Pharm.
National Community Pharmacists Association, Ronna B. Hauser, Pharm.D.
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Health Practitioner, Professional and Scientific Associations—U.S. State Medical Societies

California Medical Association, Charles W. Maas, M.D., M.P.H.
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Kansas Medical Society, Tracie Collins, M.D., M.P.H.
Maine Medical Association, Stevan Gressitt, M.D.
Massachusetts Medical Society, Bruce G. Karlin, M.D.
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Medical Association of the State of Alabama, Allan R. Goldstein, M.D.
Medical Society of New Jersey, Joseph N. Micale, M.D.
Medical Society of the District of Columbia, Kim A. Bullock, M.D.
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Minnesota Medical Association, Robert K. Meiches, M.D., M.B.A.
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South Dakota State Medical Association, E. Paul Amundson, M.D.
Tennessee Medical Association, John J. Ingram III, M.D.
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Utah Medical Association, Michelle S. McOmber, B.S., M.B.A.
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Connecticut Pharmacists Association, Peter J. Sposato, R.Ph., B.C.N.P.
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Florida Pharmacy Association, Michael A. Moné, J.D., R.Ph., FAPhA
Georgia Pharmacy Association, Larry L. Braden, B.S., R.Ph., D.Sc.
Hawaii Pharmacists Association, Ronald T. Taniguchi, Pharm.D.
Idaho State Pharmacy Association, Pam Eaton
Illinois Pharmacists Association, Norman A. Hoback, B.S.Pharm.
Indiana Pharmacists Alliance, Daniel D. Degnan III, Pharm.D., M.S.
Iowa Pharmacy Association, Thomas R. Temple, R.Ph., M.S.
Kansas Pharmacists Association, Michael Larkin
Kentucky Pharmacists Association, Patricia R. Freeman, Ph.D.
Louisiana Pharmacists Association, Randall Brooks, B.S.
Maine Pharmacy Association, Laurier A. Lamie, R.Ph.
Maryland Pharmacists Association, Matthew G. Shimoda, Pharm.D.
Massachusetts Pharmacists Association, Steven D. Geoffroy, R.Ph.
Michigan Pharmacists Association, Eric D. Roath, Pharm.D.
Minnesota Pharmacists Association, Lowell J. Anderson, D.Sc., FAPhA, FFIP
Mississippi Pharmacists Association, Kimsey O'Neal, Pharm.D.
Missouri Pharmacy Association, Ron L. Fitzwater, CAE, M.B.A.
Montana Pharmacy Association, Lori Morin, M.B.A., Pharm.D.
Nebraska Pharmacists Association, Samuel C. Augustine, Pharm.D., FAPhA
New Hampshire Pharmacists Association, Elizabeth A. Robertson, R.Ph.
New Jersey Pharmacists Association, Steve H. Zlotnick, Pharm.D.
New Mexico Pharmacists Association, William G. Troutman, Pharm.D.
North Carolina Association of Pharmacists, Stephen F. Eckel, Pharm.D., M.H.A.
North Dakota Pharmacists Association, Michael D. Schwab
Ohio Pharmacists Association, Amelia S. Bennett, R.Ph.
Oklahoma Pharmacists Association, Wiley L. Williams, J.D.
Oregon State Pharmacy Association, Joshua Free, Pharm.D., M.B.A.
Pennsylvania Pharmacists Association, George R. Haynes, Pharm.D., Ph.D.
Pharmacists Society of the State of New York, Tracy R. Russell, CAE
Pharmacy Society of Wisconsin, Susan M. Kleppin, R.Ph., FASHP
Rhode Island Pharmacists Association, John Grossomanides, Pharm.D.
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South Dakota Pharmacists Association, Leonard J. Petrik, Pharm.D.
Tennessee Pharmacists Association, Micah Cost, Pharm.D., M.S.
Texas Pharmacy Association, Eric H. Frankel, MSE, Pharm.D.
Utah Pharmacists Association, Adam W. Jones
Virginia Pharmacists Association, Marianne R. Rollings, R.Ph.
Washington D.C. Pharmacy Association, Michael J. Kim, Pharm.D.
Washington State Pharmacy Association, Jeff Rochon, Pharm.D.
West Virginia Pharmacists Association, Patty Johnston, B.S.
Wyoming Pharmacy Association, William H. Rathburn, R.Ph.

Manufacturer, Trade and Affiliated Associations

American Chemistry Council, Michael P. Walls, Esq.
American Herbal Products Association, Maged H. Sharaf, Ph.D.
American Hospital Association, John R. Combes, M.D.
Animal Health Institute, Richard A. Carnevale, V.M.D.
Association of the European Self-Medication Industry, Christelle Anquez-Traxler
Biotechnology Industry Organization, Earl S. Dye, Ph.D.
Bulk Drug Manufacturers Association, Airra Krishna Reddy, B.Sc.
Compressed Gas Association, Michael B. Tiller, B.S., B.A.
Consumer Health Products Canada, Adam C. Kingsley, B.Sc., NP, CAE
Consumer Healthcare Products Association, John Punzi, Ph.D.
Council for Responsible Nutrition, James C. Griffiths, Ph.D.
Egyptian Chamber of Pharmaceutical Industry, Abdulla Molokhia, Ph.D.
European Generic Medicines Association, Julie Marechal-Jamal, M.S.
Flavor and Extract Manufacturers Association, John H. Cox, J.D.
Food Marketing Institute, Catherine M. Polley, R.Ph.
Generic Animal Drug Alliance, Stephanie Batliner
Generic Pharmaceutical Association, David R. Gaugh, R.Ph.
Grocery Manufacturers Association, Shannon M. Cooksey, M.S., PMP
Healthcare Distribution Management Association, Karen J. Ribler
Indian Drug Manufacturers' Association, Daara B. Patel, B.Com., D.I.M.M.
International Dairy Foods Association, John T. Allan III, B.S., M.S.
International Federation of Pharmaceutical Manufacturers and Associations, Caroline Mendy
International Food Additives Council, Robert Rankin
International Generic and Biosimilar Medicines, Nicholas Cappuccino, Ph.D.
International Pharmaceutical Excipients Council of the Americas, Priscilla Zawislak
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National Association of Chain Drug Stores, Alex J. Adams, Pharm.D.
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Articles of Incorporation

In May of 1900, the USP Board of Trustees was directed by the Convention to incorporate the USP organization under the laws of the District of Columbia. Because the District of Columbia required that the majority of officers subscribing to the Certificate of Incorporation be residents, the filing was slightly delayed in order to appoint appropriate representatives. Nevertheless, the articles of incorporation were prepared, appropriately signed, and finally filed on July 11 of that year. The original certificate reads as follows:

Certification of Incorporation

This is to certify that we, whose names are hereunto subscribed, citizens of the United States, of full age, and a majority citizens of the District of Columbia, do associate ourselves together pursuant to the provisions of sections 545–552 inclusive of the Revised Statutes of the United States relating to the District of Columbia and of the Act of Congress to amend the same, approved the twenty-third day of April 1884, under the corporate name of The United States Pharmacopoeial Convention.

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USP 40

THE UNITED STATES PHARMACOPEIA

Official from May 1, 2017

FORTIETH REVISION

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Admissions

Articles Admitted to *USP 40* by Supplement

First Supplement (August 1, 2016)

GENERAL CHAPTERS

⟨507⟩ Protein Determination Procedures
⟨800⟩ Hazardous Drugs—Handling in Healthcare Settings
⟨1050.1⟩ Design, Evaluation, and Characterization of Viral Clearance Procedures
⟨1207.1⟩ Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation
⟨1207.2⟩ Package Integrity Leak Test Technologies
⟨1207.3⟩ Package Seal Quality Test Technologies
⟨1228⟩ Depyrogenation
⟨1228.1⟩ Dry Heat Depyrogenation
⟨1229.5⟩ Biological Indicators for Sterilization
⟨1229.9⟩ Physicochemical Integrators and Indicators for Sterilization
⟨1229.12⟩ New Sterilization Methods
⟨2251⟩ Adulteration of Dietary Supplements with Drugs and Drug Analogues

USP MONOGRAPHS

Abacavir and Lamivudine Tablets
Adapalene Gel
Amiodarone Hydrochloride Injection
Argatroban
Calcium Succinate
Candesartan Cilexetil Tablets
Diphenhydramine Hydrochloride and Ibuprofen Capsules
Dronedarone Hydrochloride
Dronedarone Tablets
Exemestane
Lufenuron
Palonosetron Hydrochloride
Perindopril Erbumine
Perindopril Erbumine Tablets
Rabeprazole Sodium
Sildenafil Tablets
Teniposide
Teniposide Injection
Triamcinolone Acetonide Nasal Spray
Vardenafil Hydrochloride
Zolmitriptan
Zolmitriptan Tablets

DIETARY SUPPLEMENTS

Rhodiola rosea Capsules
Rhodiola rosea Tablets
Ribose
Ubiquinol
Ubiquinol Capsules

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Second Supplement (December 1, 2016)

GENERAL CHAPTERS

⟨782⟩ Vibrational Circular Dichroism Spectroscopy
⟨1004⟩ Mucosal Drug Products—Performance Tests
⟨1029⟩ Good Documentation Guidelines
⟨1063⟩ Shear Cell Methodology for Powder Flow Testing
⟨1228.3⟩ Depyrogenation by Filtration
⟨1228.5⟩ Endotoxin Indicators for Depyrogenation
⟨1229.13⟩ Sterilization-in-Place
⟨1782⟩ Vibrational Circular Dichroism Spectroscopy—Theory and Practice

USP MONOGRAPHS

Cetirizine Hydrochloride Orally Disintegrating Tablets
Desloratadine
Desloratadine Tablets
Desloratadine Orally Disintegrating Tablets
Imipramine Pamoate Capsules
Metronidazole Extended-Release Tablets
Octreotide Acetate
Oxymorphone Hydrochloride Tablets
Oxymorphone Hydrochloride Extended-Release Tablets
Polyethylene Glycol 3350
Ropinirole Extended-Release Tablets
Zolmitriptan Orally Disintegrating Tablets

DIETARY SUPPLEMENTS

Methylcobalamin Tablets
Northern Schisandra Fruit Dry Extract

New Articles Appearing in *USP 40* That Were Not Included in *USP 39* Including Supplements

[NOTE—The articles included in this list are noted in the book with the following symbols ▲ ▲USP40. This applies to new articles as well as sections of existing items that have been revised.]

GENERAL CHAPTERS

⟨89.1⟩ Collagenase I
⟨89.2⟩ Collagenase II
⟨127⟩ Flow Cytometric Enumeration of CD34+ Cells
⟨1039⟩ Chemometrics
⟨1602⟩ Spacers and Valved Holding Chambers Used with Inhalation Aerosols—Characterization Tests
⟨1821⟩ Radioactivity—Theory and Practice
⟨1823⟩ Positron Emission Tomography Drugs—Information

USP MONOGRAPHS

Cidofovir
Cidofovir Injection
Doxercalciferol
Ixabepilone
Pemetrexed Disodium
Pemetrexed for Injection
Quetiapine Extended-Release Tablets
Selamectin
Tissue Human Amnion Chorion Membrane Dehydrated

GLOBAL HEALTH MONOGRAPHS

Chlorhexidine Gluconate Topical Gel

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DIETARY SUPPLEMENTS

Citrulline
Hesperidin
Japanese Honeysuckle Flower
Japanese Honeysuckle Flower Dry Extract
Japanese Honeysuckle Flower Powder
St. John's Wort Flowering Top Dry Extract Capsules
St. John's Wort Flowering Top Dry Extract Tablets
Sodium Ferrous Citrate
Tienchi Ginseng Root and Rhizome Dry Extract Capsules
Tienchi Ginseng Root and Rhizome Dry Extract Tablets
Tienchi Ginseng Root and Rhizome Powder Capsules
Tienchi Ginseng Root and Rhizome Powder Tablets

Articles Included in *USP 39* But Not Included in *USP 40*

GENERAL CHAPTERS

⟨21⟩ Thermometers
⟨751⟩ Metal Particles in Ophthalmic Ointments
⟨851⟩ Spectrophotometry and Light-Scattering
⟨1035⟩ Biological Indicators for Sterilization
⟨1045⟩ Biotechnology-Derived Articles
⟨1209⟩ Sterilization—Chemical and Physicochemical Indicators and Integrators

USP MONOGRAPHS

Biological Indicator for Dry-Heat Sterilization, Paper Carrier
Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier
Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions
Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers
Biological Indicator for Steam Sterilization, Paper Carrier
Biological Indicator for Steam Sterilization, Self-Contained
Chlordiazepoxide Hydrochloride for Injection
Dolasetron Mesylate Injection
Dolasetron Mesylate Tablets
Multiple Electrolytes and Dextrose Injection Type 4
Multiple Electrolytes and Invert Sugar Injection Type 1
Multiple Electrolytes and Invert Sugar Injection Type 2
Multiple Electrolytes and Invert Sugar Injection Type 3
Iopanoic Acid
Iopanoic Acid Tablets
Ipodate Sodium
Ipodate Sodium Capsules
Iophendylate
Iophendylate Injection
Mangafodipir Trisodium
Mangafodipir Trisodium Injection
Oxtriphylline Delayed-Release Tablets
Oxymorphone Hydrochloride Suppositories
Propoxyphene Hydrochloride
Propoxyphene Hydrochloride Capsules
Propoxyphene Hydrochloride and Acetaminophen Tablets
Propoxyphene Hydrochloride, Aspirin, and Caffeine Capsules
Propoxyphene Napsylate
Propoxyphene Napsylate Oral Suspension
Propoxyphene Napsylate Tablets
Propoxyphene Napsylate and Acetaminophen Tablets
Propoxyphene Napsylate and Aspirin Tablets
Sodium Chloride and Dextrose Tablets
Succinylcholine Chloride for Injection

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ANNOTATED LIST

General Notices, Monographs, General Chapters, Reagents, and Tables Affected by Changes Appearing in USP 40

Page citations refer to the pages of USP 40. Note—In the table below, if a section is new or if a subsection is added to or deleted from an existing section, it is labeled as such in parentheses after the section or subsection name. Items on this list that appear without the designation "new", "added", or "deleted" are items in which changes have been made to existing official text.

General Notices

General Notices, 3
3. Conformance to Standards; 5. Monograph Components; 6. Testing Practices and Procedures; and *8. Terms and Definitions*

General Chapters

General Tests and Assays

General Requirements for Tests and Assays

⟨1⟩ Injections and Implanted Drug Products (Parenterals)—Product Quality Tests, 67

Biological Tests and Assays

⟨89.1⟩ Collagenase I (new), 180
⟨89.2⟩ Collagenase II (new), 185
⟨127⟩ Flow Cytometric Enumeration of CD34+ Cells (new), 216
⟨151⟩ Pyrogen Test, 234
Introduction
⟨165⟩ Prekallikrein Activator (new), 241

Chemical Tests and Assays

⟨481⟩ Riboflavin Assay, 386
ASSAY
⟨531⟩ Thiamine Assay, 407
ASSAY

Physical Tests and Determinations

⟨659⟩ Packaging and Storage Requirements, 529
⟨670⟩ Auxiliary Packaging Components, 558
⟨821⟩ Radioactivity, 751

General Information

⟨1015⟩ Automated Radiochemical Synthesis Apparatus (deleted), 851
⟨1039⟩ Chemometrics (new), 963
⟨1602⟩ Spacers and Valved Holding Chambers Used with Inhalation Aerosols—Characterization Tests (new), 1988
⟨1821⟩ Radioactivity—Theory and Practice (new), 2177
⟨1823⟩ Positron Emission Tomography Drugs—Information (new), 2190

Dietary Supplements

⟨2251⟩ Screening for Undeclared Drugs and Drug Analogues, 2285

Reagents, Indicators, and Solutions

Reagent Specifications

Reagents Introduction, 2323
4. Definitions
Butylated Hydroxytoluene (new), 2341
Copper Sulfate Pentahydrate (new), 2348
Cupric Nitrate, 2348
1,1-Cyclobutanedicarboxylic Acid (new), 2349
Hydroxypropyl Cellulose (new), 2364
Isonicotinic Acid, 2366
(*R*)-(+)-Limonene (new), 2367
Lithium (new), 2368
Malonic Acid (new), 2368
D-Mannitol (new), 2369
Nickel Nitrate Hexahydrate (new), 2373
(*R*)-(−)-α-Phellandrene (new), 2378
(+)-α-Pinene (new), 2380
β-Pinene (new), 2380
Polysorbate 80 (new), 2381
Propionic Acid (new), 2383
Sabinene (new), 2386
Sodium 1-Dodecanesulfonate (new), 2391
Tetrabutylammonium Hydroxide 30-Hydrate, 2397
Tetrahydrofuran, 2398
Water (new), 2405
Water, Carbon Dioxide-Free, 2405
Water, Deaerated, 2405
Water, Organic-Free (new), 2405
Water, Particle-Free (new), 2405

Test Solutions

1 M Acetic Acid TS (new), 2411
2 M Acetic Acid TS (new), 2412
1 M Ammonium Hydroxide TS (new), 2413
2 M Ammonium Hydroxide TS (new), 2413
0.01 M Edetate Disodium TS (new), 2415
0.01 N Hydrochloric Acid TS (new), 2416
0.025 N Hydrochloric Acid TS (new), 2416
0.05 N Hydrochloric Acid TS (new), 2416
0.05 M Phosphoric Acid TS (new), 2419
0.06 M Phosphoric Acid TS (new), 2419
1 N Phosphoric Acid TS (new), 2419
20% Phosphoric Acid TS (new), 2419
1.8 N Potassium Hydroxide TS (new), 2420
45% Potassium Hydroxide TS (new), 2420
0.02 M Monobasic Potassium Phosphate TS (new), 2420
0.2 M Dibasic Potassium Phosphate TS (new), 2420
0.0025 N Sodium Hydroxide TS (new), 2421
0.02 N Sodium Hydroxide TS (new), 2421
2 N Sodium Hydroxide TS (new), 2421
2.5 N Sodium Hydroxide TS (new), 2421
10 N Sodium Hydroxide TS (new), 2421
1 M Sulfuric Acid TS (new), 2422
0.2 N Sulfuric Acid TS (new), 2422

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0.5 N Sulfuric Acid TS (new), 2422
2 N Sulfuric Acid TS (new), 2422
0.02 M Tetrabutylammonium Hydrogen Sulfate TS (new), 2422

Volumetric Solutions

2 N Acetic Acid VS, 2424
0.1 N Ammonium Thiocyanate VS, 2424
0.01 M Bismuth Nitrate VS, 2424
0.1 N Bromine VS, 2424
0.05 N Ceric Ammonium Nitrate VS, 2424
0.1 N Ceric Sulfate VS, 2424
0.1 N Cupric Nitrate VS, 2425
Cupric Tartrate, Alkaline, Solution (Fehling's Solution), 2425
0.05 M Edetate Disodium VS, 2425
0.1 M Edetate Disodium VS, 2426
0.1 N Ferric Ammonium Sulfate VS, 2426
0.1 N Ferrous Ammonium Sulfate VS, 2426
0.02 N Hydrochloric Acid VS (new), 2426
0.1 N Hydrochloric Acid VS (new), 2426
0.5 N Hydrochloric Acid VS, 2427
1 N Hydrochloric Acid VS, 2427
0.1 M Alcoholic Hydrochloric Acid VS, 2427
0.5 N Hydrochloric Acid in Methanol VS, 2427
0.01 N Iodine VS, 2427
0.05 N Iodine VS, 2427
0.1 N Iodine VS, 2427
0.01 M Lead Nitrate VS, 2428
0.01 M Lead Perchlorate VS, 2428
0.1 M Lead Perchlorate VS, 2428
0.1 N Lithium Methoxide in Chlorobenzene VS, 2428
0.02 N Lithium Methoxide in Methanol VS, 2428
0.1 N Lithium Methoxide in Methanol VS, 2429
0.1 N Lithium Methoxide in Toluene VS, 2429
0.01 M Magnesium Chloride VS, 2429
0.1 M Mercuric Nitrate VS, 2429
0.1 N Oxalic Acid VS, 2429
0.1 N Perchloric Acid in Dioxane VS, 2429
0.1 N Perchloric Acid in Glacial Acetic Acid VS, 2430
0.1 N Potassium Arsenite VS, 2430
0.1 N Potassium Bromate VS, 2430
0.1 N Potassium Bromide–Bromate VS, 2430
0.1 N Potassium Dichromate VS, 2430
0.05 M Potassium Ferricyanide VS, 2431
0.1 M Alcoholic Potassium Hydroxide VS, 2431
0.5 N Alcoholic Potassium Hydroxide VS, 2431
0.1 N Methanolic Potassium Hydroxide VS, 2431
1 N Potassium Hydroxide VS, 2431
0.05 M Potassium Iodate VS, 2431
0.1 N Potassium Permanganate VS, 2432
0.1 N Potassium Thiocyanate VS, 2432
0.002 N Silver Nitrate in Isopropyl Alcohol VS (new), 2432
0.1 N Silver Nitrate VS, 2432
0.05 N Silver Nitrate VS (new), 2432
0.05 M Sodium Arsenite VS, 2432
1 N Sodium Hydroxide VS, 2432
0.1 N Alcoholic Sodium Hydroxide VS, 2433
0.1 N Sodium Hydroxide VS (new), 2433
0.5 N Sodium Methoxide in Methanol VS, 2433
0.1 N Sodium Methoxide in Toluene VS, 2433
0.1 M Sodium Nitrite VS, 2433
0.02 M Sodium Tetraphenylboron VS, 2434
0.1 N Sodium Thiosulfate VS, 2434
Standard Dichlorophenol–Indophenol Solution, 2434
1 N Sulfuric Acid VS, 2434
0.5 N Sulfuric Acid in Alcohol VS, 2434
0.1 N Tetrabutylammonium Hydroxide VS, 2434
0.1 N Tetrabutylammonium Hydroxide in Methanol/Isopropyl Alcohol VS, 2435
0.1 M Tetramethylammonium Bromide VS, 2435
0.1 M Tetramethylammonium Chloride VS, 2435
0.1 N Titanium Trichloride VS, 2435
0.05 M Zinc Sulfate VS, 2436
0.1 M Zinc Sulfate VS (new), 2436

Chromatographic Columns

L19, 2437
L22, 2437
L40, 2437
L45, 2437
L51, 2438
L54, 2438
L56, 2438
L80, 2439
L90 (new), 2439
L91 (new), 2439

Reference Tables

Container Specifications for Capsules and Tablets

Biperiden Hydrochloride Tablets (deleted), 2444
Quetiapine Extended-Release Tablets (new), 2450
St. John's Wort Flowering Top Dry Extract Capsules (new), 2451
St. John's Wort Flowering Top Dry Extract Tablets (new), 2451
Tienchi Ginseng Root and Rhizome Powder Capsules (new), 2451
Tienchi Ginseng Root and Rhizome Powder Tablets (new), 2451
Tienchi Ginseng Root and Rhizome Dry Extract Capsules (new), 2451
Tienchi Ginseng Root and Rhizome Dry Extract Tablets (new), 2451

Description and Relative Solubility of USP and NF Articles

Doxercalciferol (new), 2471
Eucalyptus Oil (new), 2474
Ixabepilone (new), 2482
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion, 2485
Methylene Blue, 2486
Pemetrexed Disodium (new), 2492
Perindopril Erbumine (new), 2493
Sodium Succinate (new), 2503

Monographs (USP 40)

Acebutolol Hydrochloride Capsules, 2538
IDENTIFICATION
ASSAY
PERFORMANCE TESTS
Dissolution
IMPURITIES
Acetaminophen, Chlorpheniramine Maleate, and Dextromethorphan Hydrobromide Tablets, 2561
ASSAY
Acetaminophen, Chlorpheniramine Maleate, and *Dextromethorphan Hydrobromide*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
ADDITIONAL REQUIREMENTS
USP Reference Standards
Acetaminophen and Codeine Phosphate Oral Solution, 2564
ASSAY
Acetaminophen and *Codeine Phosphate*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)

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Acetaminophen and Codeine Phosphate Oral Suspension, 2565
ASSAY
Acetaminophen and *Codeine Phosphate*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
Acetaminophen, Dextromethorphan Hydrobromide, Doxylamine Succinate, and Pseudoephedrine Hydrochloride Oral Solution, 2567
ASSAY
Acetaminophen and *Dextromethorphan Hydrobromide*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
Acetaminophen and Diphenhydramine Citrate Tablets, 2569
ASSAY
Acetaminophen and *Diphenhydramine Citrate*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
Acetaminophen, Diphenhydramine Hydrochloride, and Pseudoephedrine Hydrochloride Tablets, 2570
ASSAY
Acetaminophen and *Diphenhydramine Hydrochloride*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
Acetaminophen and Pseudoephedrine Hydrochloride Tablets, 2571
ASSAY
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
Acetaminophen and Tramadol Hydrochloride Tablets, 2572
TITLE
ASSAY
OTHER COMPONENTS
Limit of p-Aminophenol (deleted)
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Tramadol Hydrochloride Extended-Release Tablets, 2575
PERFORMANCE TESTS
Dissolution
IMPURITIES
Alfuzosin Hydrochloride Extended-Release Tablets, 2626
PERFORMANCE TESTS
Dissolution
IMPURITIES
Alprazolam Extended-Release Tablets, 2644
IDENTIFICATION
Test B (added)
ASSAY
PERFORMANCE TESTS
Dissolution
ADDITIONAL REQUIREMENTS
USP Reference Standards
Alprazolam Orally Disintegrating Tablets, 2648
IDENTIFICATION
Test A and *Test B* (added)
ASSAY
PERFORMANCE TESTS
Dissolution
Amiloxate, 2719
IDENTIFICATION
Ultraviolet Absorption, Test B and *Test C* (added)
ASSAY
SPECIFIC TESTS
Acidity
Aminobenzoate Potassium, 2720
CHEMICAL INFORMATION
DEFINITION
IDENTIFICATION
Test A and *Test B*
ASSAY
IMPURITIES
Chloride and Sulfate, Chloride; *Chloride and Sulfate, Sulfate*; *Volatile Diazotizable Substances*; *Limit of Aniline and p-Toluidine*; and *Organic Impurities*
ADDITIONAL REQUIREMENTS
USP Reference Standards
Aminobenzoate Potassium Capsules, 2722
IDENTIFICATION
Test B (added)
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Aminophylline, 2735
IDENTIFICATION
Infrared Absorption, Test A and *Test B*
ASSAY
OTHER COMPONENTS
IMPURITIES
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*
Aminophylline Injection, 2737
IDENTIFICATION
Infrared Absorption, Test B and *Test C*
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*
Aminophylline Oral Solution, 2739
IDENTIFICATION
Infrared Absorption, Test A
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Aminophylline Tablets, 2742
IDENTIFICATION
Infrared Absorption, Test A and *Test B*
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*
Amlodipine and Valsartan Tablets, 2763
DEFINITION
ASSAY
PERFORMANCE TESTS
Dissolution
IMPURITIES
ADDITIONAL REQUIREMENTS
Labeling (added)
Amlodipine, Valsartan, and Hydrochlorothiazide Tablets, 2767
DEFINITION
ASSAY
PERFORMANCE TESTS
Dissolution
IMPURITIES
ADDITIONAL REQUIREMENTS
Labeling (added)
Aripiprazole Tablets, 2858
ASSAY

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PERFORMANCE TESTS
Dissolution
IMPURITIES
Ascorbic Acid Injection, 2866
IDENTIFICATION
Test C
Azathioprine Sodium for Injection, 2917
IDENTIFICATION
Test B (added)
ASSAY
SPECIFIC TESTS
Completeness of Solution (deleted)
ADDITIONAL REQUIREMENTS
Packaging and Storage
Betamethasone Valerate Cream, 3012
IDENTIFICATION
Test A and *Test B*
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Betamethasone Valerate Ointment, 3014
IDENTIFICATION
Test A and *Test B*
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Biperiden (deleted), 3028
Biperiden Hydrochloride (deleted), 3028
Biperiden Hydrochloride Tablets (deleted), 3029
Biperiden Lactate Injection (deleted), 3030
Carboplatin, 3202
DEFINITION
IDENTIFICATION
Infrared Absorption, Test A and *Test B* (added)
ASSAY
OTHER COMPONENTS
IMPURITIES
Limit of 1,1-Cyclobutanedicarboxylic Acid and *Organic Impurities*
SPECIFIC TESTS
Water Determination, Method I (deleted) and *Loss on Drying* (added)
ADDITIONAL REQUIREMENTS
Packaging and Storage
Carboplatin for Injection, 3203
IDENTIFICATION
Test B (added)
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
Packaging and Storage
Ceftriaxone for Injection, 3305
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
Packaging and Storage
Ceftriaxone Sodium, 3307
ASSAY
IMPURITIES
ADDITIONAL REQUIREMENTS
Packaging and Storage
Cidofovir (new), 3414
Cidofovir Injection (new), 3415
Cisatracurium Besylate, 3440
IMPURITIES
Organic Impurities
SPECIFIC TESTS
pH (deleted) and *Water Determination*
ADDITIONAL REQUIREMENTS
USP Reference Standards
Cisplatin, 3444
IDENTIFICATION
Infrared Absorption, Test B and *Thin-Layer Chromatography, Test C* (deleted)
ASSAY
IMPURITIES
Limit of Trichloroammineplatinate and *Limit of Transplatin*
SPECIFIC TESTS
Platinum Content
ADDITIONAL REQUIREMENTS
Packaging and Storage
Cisplatin for Injection, 3446
IDENTIFICATION
Test B (added)
ASSAY
IMPURITIES
Limit of Trichloroammineplatinate and *Limit of Transplatin*
Corticotropin Injection, 3577
ASSAY
Corticotropin for Injection, 3578
ASSAY
Repository Corticotropin Injection, 3580
ASSAY
Cyanocobalamin, 3596
ASSAY
Cysteine Hydrochloride, 3621
SPECIFIC TESTS
Loss on Drying
Demeclocycline Hydrochloride Tablets, 3647
IDENTIFICATION
ASSAY
PERFORMANCE TESTS
Dissolution
SPECIFIC TESTS
Loss on Drying (deleted)
Desipramine Hydrochloride Tablets, 3651
IDENTIFICATION
Test A and *Test B* (added)
ASSAY
IMPURITIES
Dextromethorphan, 3704
IDENTIFICATION
Test B
SPECIFIC TESTS
Melting Range or Temperature (deleted) and *Optical Rotation* (deleted)
ADDITIONAL REQUIREMENTS
USP Reference Standards
Dextromethorphan Hydrobromide, 3705
IDENTIFICATION
Test A, Test B, and *Test C* (deleted)
SPECIFIC TESTS
Optical Rotation (deleted)
ADDITIONAL REQUIREMENTS
USP Reference Standards
Diphenhydramine Hydrochloride Capsules, 3803
IMPURITIES
Diphenhydramine Hydrochloride Oral Solution, 3806
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Diphenhydramine and Phenylephrine Hydrochlorides Tablets, 3810
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Doxercalciferol (new), 3880
Doxycycline Hyclate Capsules, 3897
IDENTIFICATION
ASSAY
PERFORMANCE TESTS
Dissolution

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IMPURITIES
Organic Impurities (added)
SPECIFIC TESTS
Water Determination, Method I (deleted)
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*

Doxycycline Hyclate Tablets, 3900
IDENTIFICATION
Procedure (deleted) and *Test A* (added)
ASSAY
PERFORMANCE TESTS
Dissolution
IMPURITIES
Organic Impurities (added)
SPECIFIC TESTS
Water Determination, Method I (deleted)
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*

Dutasteride, 3924
IMPURITIES
Organic Impurities, Procedure 2

Esomeprazole Magnesium, 4057
SPECIFIC TESTS
Water Determination and *Crystallinity* (added)
ADDITIONAL REQUIREMENTS
Packaging and Storage and *Labeling*

Fenbendazole, 4151
IDENTIFICATION
IMPURITIES
Organic Impurities
ADDITIONAL REQUIREMENTS
Packaging and Storage

Fexofenadine Hydrochloride and Pseudoephedrine Hydrochloride Extended-Release Tablets, 4189
PERFORMANCE TESTS
Dissolution

Galantamine Extended-Release Capsules, 4367
ASSAY
PERFORMANCE TESTS
Dissolution
IMPURITIES

Heparin Sodium, 4475
IDENTIFICATION
Molecular Weight Determinations, Test D and *Test E*
IMPURITIES
Nucleotidic Impurities

Hydrocortisone Sodium Succinate, 4519
IDENTIFICATION
Test C

Hydroxyzine Hydrochloride, 4539
IDENTIFICATION
Infrared Absorption, Test A
ASSAY
IMPURITIES
Organic Impurities
ADDITIONAL REQUIREMENTS
USP Reference Standards

Hydroxyzine Hydrochloride Tablets, 4542
IDENTIFICATION
Test A and *Test B* (added)
ASSAY
IMPURITIES
Organic Impurities (added)
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*

Ibuprofen Oral Suspension, 4556
IDENTIFICATION
Test A and *Test B*
ASSAY
IMPURITIES
Limit of Ibuprofen Related Compound C (deleted) and *Organic Impurities* (added)
ADDITIONAL REQUIREMENTS
USP Reference Standards

Iodine Topical Solution, 4630
IDENTIFICATION
Identification Tests—General, Iodide, Test B and *Sodium, Test C* (added)

Iodine Tincture, 4631
IDENTIFICATION
Identification Tests—General, Iodide, Test B and *Sodium, Test C* (added)

Strong Iodine Tincture, 4632
IDENTIFICATION
Identification Tests—General, Iodide, Test B and *Potassium, Test C* (added)

Iothalamate Sodium Injection (deleted), 4659

Isoflurane, 4688
IMPURITIES
Chloride
SPECIFIC TESTS
Acidity or Alkalinity (added)

Ixabepilone (new), 4739

Ketorolac Tromethamine, 4753
IDENTIFICATION
Test B
IMPURITIES
Organic Impurities

Levetiracetam Extended-Release Tablets, 4812
PERFORMANCE TESTS
Dissolution

Levocetirizine Dihydrochloride, 4825
IMPURITIES
Residue on Ignition, Organic Impurities, and *Enantiomeric Purity*
SPECIFIC TESTS
Loss on Drying

Losartan Potassium and Hydrochlorothiazide Tablets, 4913
PERFORMANCE TESTS
Dissolution
ADDITIONAL REQUIREMENTS
Labeling (added)

Memantine Hydrochloride Tablets, 5000
IMPURITIES
Limit of Memantine–Lactose Adduct

Methocarbamol Tablets, 5079
IMPURITIES

Methylene Blue, 5105

Metoprolol Fumarate, 5135
DEFINITION
IDENTIFICATION
Test B
ASSAY
IMPURITIES
Organic Impurities
SPECIFIC TESTS
Melting Range or Temperature (deleted)
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*

Moexipril Hydrochloride, 5201
IMPURITIES
Organic Impurities

Moexipril Hydrochloride Tablets, 5203
IMPURITIES
Organic Impurities

Moxifloxacin Hydrochloride, 5240
ASSAY
IMPURITIES
Organic Impurities and *Enantiomeric Purity* (added)
SPECIFIC TESTS
Optical Rotation (deleted)

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ADDITIONAL REQUIREMENTS
USP Reference Standards
Nicotine Polacrilex, 5347
DEFINITION
IDENTIFICATION
Infrared Absorption for Nicotine, Test A and *Infrared Absorption for Polacrilex, Test B*
ASSAY
SPECIFIC TESTS
Water Determination (deleted), *Loss on Drying* (added), and *Nicotine Release*
ADDITIONAL REQUIREMENTS
Packaging and Storage
Olopatadine Hydrochloride, 5425
IDENTIFICATION
Infrared Absorption, Test A
ASSAY
IMPURITIES
Organic Impurities
Olopatadine Hydrochloride Ophthalmic Solution, 5426
IDENTIFICATION
Test B (added)
ASSAY
IMPURITIES
Limit of Early Eluting Impurities and *Limit of Late Eluting Impurities*
Pemetrexed Disodium (new), 5588
Pemetrexed for Injection (new), 5590
Penicillin G Procaine, 5607
CHEMICAL INFORMATION
ASSAY
SPECIFIC TESTS
Content of Penicillin G and Procaine, Sterility Tests, and *pH*
ADDITIONAL REQUIREMENTS
Packaging and Storage and *USP Reference Standards*
Pentazocine and Acetaminophen Tablets, 5625
ASSAY
Pentazocin and *Acetaminophen*
IMPURITIES
4-Aminophenol in Acetaminophen-Containing Drug Products (added)
Phenoxybenzamine Hydrochloride Capsules, 5669
IDENTIFICATION
Test A and *Test B* (added)
ASSAY
PERFORMANCE TESTS
Dissolution and *Uniformity of Dosage Units*
Phenylephrine Hydrochloride Tablets, 5685
IMPURITIES
ADDITIONAL REQUIREMENTS
USP Reference Standards
Prompt Phenytoin Sodium Capsules (deleted), 5696
Pioglitazone and Metformin Hydrochloride Tablets, 5720
IDENTIFICATION
Ultraviolet Absorption, Test A
ASSAY
Polymyxin B Sulfate, 5750
IMPURITIES
Organic Impurities
SPECIFIC TESTS
Composition of Polymyxins
Potassium Gluconate, 5777
IDENTIFICATION
Test B
Potassium Gluconate Oral Solution, 5778
IDENTIFICATION
Test A
Potassium Gluconate Tablets, 5779
IDENTIFICATION
Test B
Prochlorperazine Maleate, 5849
DEFINITION
IDENTIFICATION
Test A and *Test B* (added)
ASSAY
IMPURITIES
Organic Impurities
Propafenone Hydrochloride Extended-Release Capsules, 5876
PERFORMANCE TESTS
Dissolution
IMPURITIES
Organic Impurities and *Content of Propafenone Related Compound A*
ADDITIONAL REQUIREMENTS
Labeling (added)
Protamine Sulfate, 5902
ASSAY
IMPURITIES
Elemental Impurities (deleted)
ADDITIONAL REQUIREMENTS
USP Reference Standards
Protamine Sulfate Injection, 5903
ASSAY
Quetiapine Extended-Release Tablets (new), 5937
Ribavirin Capsules, 5996
PERFORMANCE TESTS
Dissolution
Riboflavin Tablets, 6002
ASSAY
Ringer's Injection, 6019
IDENTIFICATION
Identification Tests—General, Test A; Sodium, Test B (added); and *Potassium, Test C* (added)
Secobarbital Sodium Injection (deleted), 6132
Secobarbital Sodium for Injection (deleted), 6132
Secobarbital Sodium and Amobarbital Sodium Capsules (deleted), 6133
Selamectin (new), 6133
Sodium Citrate and Citric Acid Oral Solution, 6186
IDENTIFICATION
Test A and *Identification Tests—General, Test D*
ASSAY
Sodium, Sodium Citrate, and *Citric Acid*
Sodium Hypochlorite Solution, 6192
IDENTIFICATION
Test B and *Test C* (deleted)
ASSAY
Sulfadoxine and Pyrimethamine Tablets, 6263
IDENTIFICATION
Test A
ASSAY
Tamsulosin Hydrochloride Capsules, 6320
ASSAY
Procedure 8 and *Procedure 10* (added)
PERFORMANCE TESTS
Dissolution
Tetrahydrozoline Hydrochloride, 6410
DEFINITION
IDENTIFICATION
Infrared Absorption, Test A and *Test B*
ASSAY
IMPURITIES
Ordinary Impurities (deleted) and *Organic Impurities* (added)
ADDITIONAL REQUIREMENTS
Packaging and Storage
Thiamine Hydrochloride Tablets, 6432
ASSAY
Tinidazole, 6483
DEFINITION

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IDENTIFICATION
Test B and *Test C* (deleted)
ASSAY
IMPURITIES
Organic Impurities
SPECIFIC TESTS
Melting Range or Temperature (deleted)
Tissue Human Amnion Chorion Membrane Dehydrated (new), 6486
Valine, 6642
IMPURITIES
Related Compounds
ADDITIONAL REQUIREMENTS
USP Reference Standards

Monographs (Global Health)

Chlorhexidine Gluconate Topical Gel (new), 6779

Monographs (Dietary Supplements)

Ashwagandha Root, 6800
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test A and *HPLC, Test B*
COMPOSITION
ADDITIONAL REQUIREMENTS
USP Reference Standards
Powdered Ashwagandha Root, 6802
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test A and *HPLC, Test B*
COMPOSITION
ADDITIONAL REQUIREMENTS
USP Reference Standards
Powdered Ashwagandha Root Extract, 6804
DEFINITION
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test A and *HPLC, Test B*
COMPOSITION
ADDITIONAL REQUIREMENTS
USP Reference Standards
Citrulline (new), 6907
Crypthecodinium cohnii Oil, 6912
IDENTIFICATION
Curcuminoids, 6917
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test A and *HPLC, Test B*
COMPOSITION
CONTAMINANTS
Botanical Extracts (added)
Hesperidin (new), 7048
Japanese Honeysuckle Flower (new), 7055
Japanese Honeysuckle Flower Dry Extract (new), 7058
Japanese Honeysuckle Flower Powder (new), 7060
Rutin, 7164
IMPURITIES
Quercetin and Other Related Compounds
St. John's Wort Flowering Top, 7166
TITLE
DEFINITION
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test B
COMPOSITION
Content of Hypericin and Pseudohypericin and *Content of Hyperforin*
SPECIFIC TESTS
Loss on Drying
St. John's Wort Flowering Top Powder, 7168
TITLE
DEFINITION
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test B
COMPOSITION
Content of Hypericin and Pseudohypericin and *Content of Hyperforin*
SPECIFIC TESTS
Loss on Drying
St. John's Wort Flowering Top Dry Extract, 7169
TITLE
DEFINITION
IDENTIFICATION
HPTLC for Articles of Botanical Origin, Test A and *Thin-Layer Chromatography, Test B*
COMPOSITION
Content of Hypericin and Pseudohypericin and *Content of Hyperforin*
St. John's Wort Flowering Top Dry Extract Capsules (new), 7172
St. John's Wort Flowering Top Dry Extract Tablets (new), 7173
Sodium Ferrous Citrate (new), 7195
Tienchi Ginseng Root and Rhizome Powder Capsules (new), 7218
Tienchi Ginseng Root and Rhizome Powder Tablets (new), 7219
Tienchi Ginseng Root and Rhizome Dry Extract Capsules (new), 7223
Tienchi Ginseng Root and Rhizome Dry Extract Tablets (new), 7225
Turmeric, 7228
DEFINITION
IDENTIFICATION
Test A and *Test B*
COMPOSITION
Powdered Turmeric, 7230
DEFINITION
IDENTIFICATION
Test A and *Test B*
COMPOSITION
SPECIFIC TESTS
Articles of Botanical Origin, Foreign Organic Matter (deleted)
Powdered Turmeric Extract, 7232
IDENTIFICATION
Test A and *Test B*
COMPOSITION

⟨1032⟩ DESIGN AND DEVELOPMENT OF BIOLOGICAL ASSAYS

1. INTRODUCTION

1.1 Purpose and Scope

General chapter *Design and Development of Biological Assays* ⟨1032⟩ presents methodology for the development of bioassay procedures that have sound experimental design, that provide data that can be analyzed using well-founded statistical principles, and that are fit for their specific use.

General chapter ⟨1032⟩ is one of a group of five general chapters that focus on relative potency assays, in which the activity of a Test material is quantified by comparison to the activity of a Standard material. However, many of the principles can be applied to other assay systems.

This general chapter is intended to guide the design and development of a bioassay for a drug substance or product intended for commercial distribution. Although adoption of this chapter's recommended methods may be resource intensive during assay development, early implementation can yield benefits. Lastly, the perspectives and methods described herein are those recommended from among the many alternatives which contemporary bioassay theory and practice offers.

Focus on Relative Potency

Because of the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically *similar*, *statistical similarity* (a consequence of the Test and Standard similarity) should be present, and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unitless measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations. For the purpose of the relative comparison of Test to Standard, the potency of the Standard is usually assigned a value of 1 (or 100%). The Standard can be a material established as such by a national (e.g., USP) or international (e.g., WHO) organization, or it could be an internal Standard.

1.2 Audience

This chapter is intended for both the practicing bioassay analyst and the statistician who are engaged in developing a bioassay. The former will find guidance for implementing bioassay structure and methodology to achieve analytical goals while reliably demonstrating the biological activity of interest, and the latter will gain insights regarding the constraints of biology that can prove challenging to balance with a rigorous practice of statistics.

2. BIOASSAY FITNESS FOR USE

To evaluate whether an assay is fit for use, analysts must specify clearly the purpose(s) for performing the bioassay. Common uses for a bioassay include lot release of drug substance (active pharmaceutical ingredient) and drug product; assessment of stability; qualification of Standard and other critical reagents; characterization of process intermediates and formulations; characterization of contaminants and degradation products; and support of changes in the product production process. The relative accuracy, specificity, precision, and robustness requirements may be different for each of these potential uses. It is a good strategy to develop and validate a bioassay to support multiple intended uses; for example, a bioassay primarily developed for batch release may serve other purposes. Decisions about fitness for use are based on scientific and statistical considerations, as well as practical considerations such as cost, turnaround time, and throughput requirements for the assay.

When assays are used for lot release, a linear-model bioassay may allow sufficient assessment of similarity. For bioassays used to support stability, comparability, to qualify reference materials or critical reagents, or in association with changes in the production or assay processes, it is generally useful to assess similarity using the entire concentration–response curve, including the asymptotes (if present).

2.1 Process Development

Bioassays are generally required in the development and optimization of product manufacturing, including formulation and scale-up processes. Bioassays can be used to evaluate purification strategies, optimize product yield, and measure product stability. Because samples taken throughout the process are often analyzed and compared, sample matrix effects that may affect assay response should be carefully studied to determine an assay's fitness for use. For relative potency measures, the Standard

material may require dilution into a suitable matrix for quantitation. The bioassay's precision and accuracy should be sufficient for measuring process performance or for assessing and comparing the stability of candidate formulations.

2.2 Process Characterization

Bioassays may be performed to assess the effect on drug potency associated with different stages of drug manufacture or with changes in the manufacturing process (e.g., to demonstrate product equivalence before and after process changes are made). Bioassays used in this type of application may be qualitative or quantitative.

2.3 Product Release

Bioassays are used to evaluate the potency of the drug before commercial product release. To the extent possible, the assay should reflect or mimic the product's known or intended mechanism of action. If the bioassay does not include the functional biology directly associated with the mechanism of action, it may be necessary to demonstrate a relationship between the bioassay's estimated potency determinations and those of some other assay that better or otherwise reflects putative functional activity.

For product-release testing, product specifications are established to define a minimum or range of potency values that are acceptable for product. The precision of the reportable value from the bioassay must support the number of significant digits listed in the specification (see general chapter *Biological Assay Validation* ⟨1033⟩), and, in conjunction with relative accuracy, support the specification range. In order to meet these specifications, manufacturing quality control will have sufficiently narrow product release specifications in order to accommodate any loss of activity due to instability and uncertainty in the release assay.

2.4 Process Intermediates

Bioassay assessment of process intermediates can provide information regarding specificity. Formulation and fill strategies may rely on bioassays in order to ensure that drug product, including that in final container, will meet its established specifications. For example, unformulated bulk materials may be held and evaluated for potency. Bulks may be pooled with other bulk lots, diluted, or reworked based on the potency results. For these types of applications, the bioassay must be capable of measuring product activity in different matrices. In some cases, a separate Standard material is made and is used to calculate relative potency for the process intermediate.

2.5 Stability

The potency assay may be used to assess biotechnology and vaccine product stability. Information from stability studies, performed during development under actual and/or accelerated or stressed storage conditions, may be used to establish shelf life duration as well as to identify and estimate degradation products and degradation rates. Post licensure stability studies may be used to monitor product stability. Knowledge of both short-term and long-term variability of the bioassay is important to assure an acceptable level of uncertainty in potency measures obtained.

2.6 Qualification of Reagents

The quantitative characterization of a new Standard requires an accurate and precise measurement of the new Standard's biological activity. This measurement is used either to establish that the new Standard lot is equivalent to the previous lot or to assign it a label potency to which Test samples can be compared. Additional replication (beyond routine testing) may be required to achieve greater precision in the potency measurement of the new Standard material. Additionally, the bioassay may be used to qualify a cell culture reagent such as fetal bovine serum. The fitness for use in such cases is tied to the ability of the assay to screen reagent lots and to ensure that lots that may bias or compromise the relative potency measurements are not accepted.

2.7 Product Integrity

Biotechnology, biological, and vaccine products may contain a population of heterogeneous material, including the intended predominant product material. Some process impurities and degradation products may be active, partially active, inactive in, or antagonistic to, the response measured in the bioassay. For product variants or derivatives for which changes in structure or relative composition may be associated with subtle yet characteristic changes in the bioassay response (e.g., change in slope or asymptote), the bioassay may be useful in the detection and measurement of these variants or derivatives. Studies that identify characteristic changes associated with variants of the intended product help ensure consistent product performance. Whenever practical, the bioassay should be accompanied by orthogonal methods that are sensitive to product variants, process impurities, and/or degradation products.

3. BIOASSAY FUNDAMENTALS

3.1 In Vivo Bioassays

In vivo potency assays are bioassays in which sets of dilutions of the Standard and Test materials are administered to animals and the concentration–response relationships are used to estimate potency. For some animal assays, the endpoint is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoeitin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has substantially diminished. Cost, low throughput, ethical, and other practical issues argue against the use of animal bioassays. Regulatory agencies have encouraged the responsible limitation of animal use whenever possible (see The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities; February 2004). When in vitro activity is not strongly associated with in vivo activity (e.g., EPO), the combination of an in vitro cell-based assay and a suitable physicochemical method (e.g., IEF, glycan analysis) may substitute for in vivo assays. However, a need for in vivo assays may remain when in vitro assays cannot detect differences that are critical in regard to a drug's intended biological function.

Animals' physiological responses to biological drugs (including vaccines) may predict patients' responses. Selection of animal test subjects by species, strain, gender, and maturity or weight range is guided by the goal of developing a representative and sensitive model with which to assess the activity of Test samples.

Some assay methods lend themselves to the use of colony versus naive animals. For example, pyrogen and insulin testing benefit from using experienced colony rabbits that provide a reliable response capacity. If animals recently introduced to the colony fail to respond as expected after several administrations of a compound, they should be culled from the colony so they do not cause future invalid or indeterminate assay results. In the case of assaying highly antigenic compounds for pyrogens, however, naive animals should be used to avoid generating inaccurate or confounded results. Other colony advantages include common controlled environmental conditions (macro/room, and micro/rack), consistent feeding schedule, provision of water, and husbandry routine.

Historical data including colony records and assay data can be used to identify factors that influence assay performance. The influence of biasing factors can be reduced by applying randomization principles such as distribution of weight ranges across dose groups, group assignments from shipping containers to different cages, or use of computer-generated or deck patterns for injection/dosing. A test animal must be healthy and have time to stabilize in its environment to be suitable for use in a bioassay. Factors that combine to influence an animal's state of health include proper nutrition, hydration, freedom from physical and psychological stressors, adequate housing sanitization, controlled light cycle (diurnal/nocturnal), experienced handling, skillful injections and bleedings, and absence of noise or vibration. Daily observation of test animals is essential for maintenance of health, and veterinary care must be available to evaluate issues that have the potential to compromise the validity of bioassay results.

3.2 Ex Vivo Bioassays

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test sample. In the case of cytokines, the majority of assays use cells from the hematopoietic system or subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells.

Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of in vivo assays to minimize assay variability and bias. The level of effort to manage bias (e.g., via randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

3.3 In Vitro (Cell-Based) Bioassays

Bioassays using cell lines that respond to specific ligands or infectious agents can be used for lot-release assays. These cell lines can be derived from tumors, immortalized as factor-dependent cell lines, or engineered cell lines transfected with appropriate receptors. Additionally, nontransformed cell lines which can be maintained over a sufficient number of passages (e.g., fibroblasts) may also be used. Regardless of cell line, there is an expectation of adequately equivalent potency response through some number of continuous passages. Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response, stable expression of re-

ceptors and signaling mechanisms, and longer stability. The cellular responses to the protein of interest depend on the drug's MOA and the duration of exposure. Such responses include cell proliferation, cell killing, antiviral activity, differentiation, cytokine/mediator secretion, and enzyme activation. Assays involving these responses may require incubation of the cells over several days, during which time contamination, uneven evaporation, or other location effects may arise. Comparatively rapid responses based on an intracellular signaling mechanism—such as second messengers, protein kinase activation, or reporter gene expression—have proven acceptable to regulatory authorities. Lastly, most cell lines used for bioassays express receptors for multiple cytokines and growth factors. This lack of specificity may not be detrimental if the Test sample's specificity is demonstrated.

Cell-based bioassay design should reflect knowledge of the factors that influence the response of the cells to the active analyte. Response variability is often reflected in parameters such as slope, EC_{50} of the concentration–response curve, or the response range (maximum minus minimum response). Even though relative potency methodology minimizes the effects on potency estimates of variation in these parameters among assays, and among blocks within an assay, such response variability can make an assay difficult to manage (i.e., it may be difficult to assess system suitability). Hence, while assay development should be focused primarily on the properties of potency, efforts to identify and control variation in the concentration–response relationship are also appropriate. For blocked assays (e.g., multiple cell culture plates in an assay) with appreciable variation in curve shape among blocks, an analysis that does not properly include blocks will yield inflated estimates of within-assay variation, making similarity assessment particularly difficult. Two strategies are available for addressing variation among blocks: one, a laboratory effort to identify and control sources of variation and two, a statistical effort to build and use a blocked design and analysis. Combining these strategies can be particularly effective.

The development of a cell-based bioassay begins with the selection or generation of a cell line. An important first step when developing a cell-based assay to assess a commercial product is to verify that the cell line of interest is not restricted to research use only. To ensure an adequate and consistent supply of cells for product testing, a cell bank should be generated if possible. To the extent possible, information regarding functional and genetic characteristics of the bioassay's cell line should be documented, including details of the cell line's history from origin to banking. For example, for a recombinant cell line this might include the identification of the source of the parental cell line (internal cell bank, external repository, etc.), of the DNA sequences used for transfection, and of the subsequent selection and functional testing regimen that resulted in selection of the cell line. Ideally, though not always practical, sufficient information is available to permit recreation of a similar cell line if necessary. Pertinent information may include identity (e.g., isoenzyme, phenotypic markers, genetic analysis); morphology (e.g., archived photographic images); purity (e.g., mycoplasma, bacteria, fungus and virus testing); cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions); thaw viability (immediately after being frozen and after time in storage); growth characteristics (e.g., cell doubling times); and functional stability (e.g., ploidy).

Cell characterization and vigilance regarding aspects of assay performance that reflect on cell status are necessary to ensure the quality and longevity of cell banks for use in the QC environment. The general health and metabolic state of the cells at the time of bioassay can substantially influence the test results. After a cell line has been characterized and is ready for banking, analysts typically prepare a two-tiered bank (Master and Working). A Master Cell Bank is created as the source for the Working Cell Bank. The Working Cell Bank is derived by expansion of one or more vials of the Master Cell Bank. The size of the banks depends on the growth characteristics of the cells, the number of cells required for each assay, and how often the assay will be performed. Some cells may be sensitive to cryopreservation, thawing, and culture conditions, and the banks must be carefully prepared and characterized before being used for validation studies and for regular use in the QC laboratory.

There follow factors that may affect bioassay response and the assessment of potency, that are common to many cell-based bioassays: cell type (adherent or nonadherent); cell thawing; plating density (at thaw and during seed train maintenance) and confluence (adherent cells); culture vessels; growth, staging, and assay media; serum requirements (source, heat inactivation, gamma irradiation); incubation conditions (temperature, CO_2, humidity, culture times from thaw); cell harvesting reagents and techniques (for adherent cells, method of dissociation); cell sorting; cell counting; determination of cell health (growth rate, viability, yield); cell passage number and passaging schedule; cell line stability (genetic, receptor, marker, gene expression level); and starvation or stimulation steps. This list is not exhaustive, and analysts with comprehensive understanding and experience with the cell line should be involved during assay development. These experienced individuals should identify factors that might influence assay outcomes and establish strategies for an appropriate level of control whenever possible.

3.4 Standard

The Standard is a critical reagent in bioassays because of the necessity to have a reliable material to which a Test preparation can be quantitatively compared. The Standard may be assigned a unitage or specific activity that represents fully (100%) potent material. Where possible, a Standard should be representative of the samples to be tested in the bioassay. Testing performed to qualify a Standard may be more rigorous than the routine testing used for lot release.

A Standard must be stored under conditions that preserve its full potency for the intended duration of its use. To this end, the Standard may be stored under conditions that are different from the normal storage of the drug substance or drug product. These could include a different temperature (e.g., −70° or −20°, instead of 2°–8°), a different container (e.g., plastic vials instead of syringes), a different formulation (e.g., lyophilizable formulation or the addition of carrier proteins such as human serum albumin, stabilizers, etc.). The Standard material should be tested for stability at appropriate intervals. System suitability

criteria of the bioassay such as maximum or background response, EC_{50} slope, or potency of assay control may be used to detect change in the activity of the Standard. Accelerated stability studies can be performed to estimate degradation rates and establish recognizable characteristics of Standard instability.

At later stages in clinical development, the Standard may be prepared using the manufacturing process employed in pivotal clinical trials. If the Standard formulation is different from that used in the drug product process, it is important to demonstrate that the assay's assessment of similarity and estimate of potency is not sensitive to the differences in formulation. An initial Standard may be referred to as the *Primary Standard.* Subsequent Standards can be prepared using current manufacturing processes and can be designated *Working Standards.* Separate SOPs may be required for establishing these standards for each product. Bias in potency measurements sometimes can arise if the activity of the Standard gradually changes. Also, loss of similarity may be observed if, with time, the Standard undergoes changes in glycosylation. It is prudent to archive aliquots of each Standard lot for assessment of comparability with later Standards and for the investigation of assay drift.

4. STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS

The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample.

4.1 Data

Fundamentally, there are two bioassay data types: quantitative and quantal (categorical). Quantitative data can be either continuous (not limited to discrete observations; e.g., collected from an instrument), count (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titers). Quantal data are often dichotomous; for example, life/death in an animal response model or positivity/negativity in a plate-based infectivity assay that results in destruction of a cell monolayer following administration of an infectious agent. Quantitative data can be transformed to quantal data by selecting a threshold that distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, as by adding (or subtracting) a measure of uncertainty (such as two or three times the standard deviation of negative control responses) to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information.

4.2 Assumptions

A key assumption for the analysis of most bioassays is that the Standard and Test samples contain the same effective analyte or population of analytes and thus may be expected to behave similarly in the bioassay. This is termed *similarity.* As will be shown in more detail in the general chapter *Analysis of Biological Assays* ⟨1034⟩ for specific statistical models, biological similarity implies that statistical similarity is present (for parallel-line and parallel-curve models, the Standard and Test curves are parallel; for slope-ratio models, the Standard and Test lines have a common intercept). The reverse is not true. Statistical similarity (parallel lines, parallel curves, or common intercept, as appropriate) does not ensure biological similarity. However, failure to satisfy statistical similarity may be taken as evidence against biological similarity. The existence of a Standard–Test sample pair that passes the assessment of statistical similarity is thus a necessary but not sufficient condition for the satisfaction of the key assumption of biological similarity. Biological similarity thus remains, unavoidably, an assumption. Departures from statistical similarity that are consistent in value across replicate assays may be indicative of matrix effects or of real differences between Test and Standard materials. This is true even if the departure from statistical similarity is sufficiently small to support determination of a relative potency.

In many assays multiple compounds will yield similar concentration–response curves. It may be reasonable to use a biological assay system to describe or even compare response curves from different compounds. But it is not appropriate to report relative potency unless the Standard and Test samples contain only the same active analyte or population of analytes. Biological products typically exhibit lot-to-lot variation in the distribution of analytes (i.e., most biological products contain an intended product and, at acceptably low levels, some process contaminants that may be active in the bioassay). Assessment of similarity is then, at least partially, an assessment of whether the distribution of analytes in the Test sample is close enough to that of the distribution in the Standard sample for relative potency to be meaningful; that is, the assay is a comparison of like to like. When there is evidence (from methods other than the bioassay) that the Standard and Test samples do not contain the same active compound(s), the assumption of biological similarity is not satisfied, and it is not appropriate to report relative potency.

Other common statistical assumptions in the analysis of quantitative bioassays are constant variance of the responses around the fitted model (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation* for further discussion), normally distributed residuals (a residual is the difference between an observed response and the response predicted by the model), and independence of the residuals.

Constant variance, normality, and independence are interrelated in the practice of bioassay. For bioassays with a quantitative response, a well-chosen data transformation may be used to obtain approximately constant variance and a nearly normal

General Chapters

distribution of residuals. Once such transformation has been imposed, the remaining assumption of independence then remains to be addressed via reflection of the assay design structure in the analysis model. Independence of residuals is important for assessing system and sample suitability.

4.3 Variance Heterogeneity, Weighting, and Transformation

Simple analysis of quantitative bioassay data requires that the data be approximately normally distributed with near constant variance across the range of the data. For linear and nonlinear regression models, the variance referred to here is the residual variance from the fit of the model. Constant variance is often not observed; *variance heterogeneity* may manifest as an increase in variability with increase in response. If the variances are not equal but the data are analyzed as though they are, the estimate of relative potency may still be reasonable; however, failure to address nonconstant variance around the fitted concentration–response model results in an unreliable estimate of within-assay variance. Further, the assessment of statistical similarity may not be accurate, and standard errors and confidence intervals for all parameters (including a Fieller's Theorem-based interval for the relative potency) should not be used. Confidence intervals for relative potency that combine potency estimates from multiple assays may be erroneous if within-assay error is used for confidence interval calculation.

Constancy of variance may be assessed by means of residual plots, Box-Cox (or power law) analysis, or Levene's test. With Levene's test, rather than relying on the p value, change in the statistic obtained is useful as a basis for judging whether homogeneity is improved or worsened. Variance is best assessed on a large body of assay data. Using only the variance among replicates from the current assay is not appropriate, because there are too few data to properly determine truly representative variances specific to each concentration. Data on variance is sparse during development; it is prudent to re-assess variance during validation and to monitor it periodically during ongoing use of the assay.

Two methods used to mitigate variance heterogeneity are transformation and weighting. Lack of constant variance can be addressed with a suitable transformation. Additionally, transformation can improve the normality of residuals and the fit of some statistical models to the data. A transformation should be chosen for an assay system during development, checked during validation, used consistently in routine assay practice, and checked periodically. Bioassay data are commonly displayed with log-transformed concentration; slope-ratio assays are displayed with concentration on the original scale.

Transformation may be performed to the response data as well as to the concentration data. Common choices for a transformation of the response include log, square root (for counts), reciprocal, and, for count data with known asymptotes, logit of the percent of maximum response. Log transformations are commonly used, as they may make nearly linear a useful segment of the concentration–response relationship, and because of the ease of transforming back to the original scale for interpretation. A log–log fit may be performed on data exhibiting nonlinear behavior. Other alternatives are available; i.e., data may be transformed by the inverse of the *Power of the Mean* (POM) function. A POM coefficient of k = 2 corresponds to a log transformation of the data. For further discussion of relationships between log-transformed and untransformed data, see *Appendix* in the general chapter *Biological Assay Validation* ⟨1033⟩.

Note that transformation of the data requires re-evaluation of the model used to fit the data. From a statistical perspective there is nothing special about the original scale of measurement; any transformation that improves accordance with assumptions is acceptable. Analysts should recognize, however, that transformations, choice of statistical model, and choice of weighting scheme are interrelated. If a transformation is used, that may affect the choice of model. That is, transforming the response by a log or square root, for example, may change the shape of the response curve, and, for a linear model, may change the range of concentrations for which the responses are nearly straight and nearly parallel.

For assays with non-constant variance, a weighted analysis may be a reasonable option. Though weighting cannot address lack of residual normality, it is a valid statistical approach to placing emphasis on more precise data. Ideally, weights may be based on the inverse of the predicted within-assay (or within-block) variance of each response where the predictors of variance are independent of responses observed in a specific assay.

In practice, many bioassays have relatively large variation in log EC_{50} (compared to the variation in log relative potency) among assays (and sometimes among blocks within assay). If not addressed in the variance model, this variation in log EC_{50} induces what appears to be large variation in response near the mean log EC_{50}, often yielding too-low weights for observations near the EC_{50}.

If the assay is fairly stable (low variability in EC_{50}), an alternative is to look at variance as a function of concentration. While not ideal, an approach using concentration-dependent variances may be reasonable when the weights are estimated from a large number of assays, the variances are small, any imbalance in the number of observations across concentrations is addressed in the variance model, and there are no unusual observations (outliers). This possibility can be examined by plotting the response variance at each concentration (preferably pooled across multiple assays) against concentration and then against a function of concentration (e.g., concentration squared). Variance will be proportional to the function of concentration where this plot approximates a straight line. The apparent slope of this line is informative, in that a horizontal line indicates no weighting is needed. If a function that yields a linear plot can be found, then the weights are taken as proportional to the reciprocal of that function. There may be no such function, particularly if the variation is higher (or lower) at both extremes of the concentration range studied.

Whether a model or historical data are used, the goal is to capture the relative variability at each concentration. It is not necessary to assume that the absolute level of variability of the current assay is identical to that of the data used to determine

the weighting, but only that the ratios of variances among concentrations are consistent with the historical data or the data used to determine the variance function.

Appropriate training and experience in statistical methods are essential in determining an appropriate variance-modeling strategy. Sources of variability may be misidentified if the wrong variance model is used. For example, data may have constant variation throughout a four-parameter logistic concentration–response curve but can also have appreciable variation in the EC_{50} parameter from block to block within the assay, or from assay to assay. If the between-block or between-assay variability is not recognized, this assay can appear to have large variation in the response for concentrations near the long-term average value of the EC_{50}. A weighted model with low weights for concentrations near the EC_{50} would misrepresent a major feature of such an assay system.

4.4 Normality

Many statistical methods for the analysis of quantitative responses assume normality of the residuals. If the normality assumption is not met, the estimate of relative potency and its standard error may be reasonable, but suitability tests and a confidence interval for the relative potency estimate may be invalid. Most methods used in this chapter are reasonably robust to departures from normality, so the goal is to detect substantial nonnormality. During assay development, in order to discover substantial departure from normality, graphical tools such as a normal probability plot or a histogram (or something similar like stem-and-leaf or box plots) of the residuals from the model fit may be used. The histogram should appear unimodal and symmetric. The normal probability plot should approximate a straight line; a normal probability plot that is not straight (e.g., curved at one end, both ends, or in the middle) indicates the presence of nonnormality. A pattern about a straight line is an indication of nonnormality. Nonnormal behavior may be due to measurements that are log normal and show greater variability at higher levels of response. This may be seen as a concave pattern in the residuals in a normal plot.

Statistical tests of normality may not be useful. As per the previous discussion of statistical testing of constancy of variance, change of the value of a normality test statistic, rather than reliance on a p value, is useful for judging whether normality is improved or worsened. As for variance assessment, evaluate normality on as large a body of assay data as possible during development, re-assess during validation, and monitor periodically during ongoing use of the assay. Important departures from normality can often be mitigated with a suitable transformation. Failure to assess and mitigate important departure from normality carries the risks of disabling appropriate outlier detection and losing capacity to obtain reliable estimates of variation.

4.5 Linearity of Concentration–Response Data

Some bioassay analyses assume that the shape of the concentration–response curve is a straight line or approximates a straight line over a limited range of concentrations. In those cases, a linear-response model may be assessed to determine if it is justified for the data in hand. Difference testing methods for assessing linearity face the same problems as do difference testing methods applied to parallelism—more data and better precision make it more likely to detect nonlinearity. Because instances in which lack of linearity does not affect the potency estimate are rare, analysts should routinely assess departure from linearity if they wish to use a linear-response model to estimate potency.

If an examination of a data plot clearly reveals departure from linearity, this is sufficient to support a conclusion that linearity is not present. High data variability, however, may mask departure from linearity. Thus a general approach for linearity can conform to that for similarity, developed more elaborately in section 4.7 *Suitability Testing, Implementing Equivalence Testing for Similarity (parallelism)*.

(1) Specify a measure of departure from linearity which can either combine across samples or be sample specific. Possibilities include the nonlinearity sum of squares or quadratic coefficients.

(2) Use one of the four approaches in *Step 2* of *Implementing Equivalence Testing for Similarity (parallelism)* to determine, during development, a range of acceptable values (acceptance interval) for the measure of nonlinearity.

(3) Determine a 90% two-sided confidence interval on the measure on nonlinearity, following the Two One-Sided Test (TOST) procedure, and compare the result to the acceptance interval as determined in (2).

Often a subset of the concentrations measured in the assay will be selected in order to establish a linear concentration–response curve. The subset may be identified graphically. The concentrations at the extreme ends of the range should be examined carefully as these often have a large impact on the slope and calculations derived from the slope. If, in the final assay, the intent is to use only concentrations in the linear range, choose a range of concentrations that will yield parallel straight lines for the relative potencies expected during routine use of the assay; otherwise, the assay will fail parallelism tests when the potency produces assay response values outside the linear range of response. When potency is outside the linear range, it may be appropriate to adjust the sample concentration based on this estimated potency and test again in order to obtain a valid potency result. The repeat assays together with the valid assays may generate a biased estimate of potency because of the selective process of repeating assays when the response is in the extremes of the concentration–response curve.

The problem is more complex in assays where there is even modest variation in the shape or location of the concentration–response curve from run to run or from block to block within an assay. In such assays it may be appropriate to choose subsets for each sample in each assay or even in each block within an assay. Note that a fixed-effects model will mask any need for different subsets in different blocks, but a mixed-effects model may reveal and accommodate different subsets in different blocks (see section 4.9 *Fixed and Random Effects in Models of Bioassay Response*).

Additional guidance about selection of data subset(s) for linear model estimation of relative potency includes the following: use at least three, and preferably four, adjacent concentrations; require that the slope of the linear segment is sufficiently steep; require that the lines fit to Standard and Test samples are straight; and require that the fit regression lines are parallel. One way to derive a steepness criterion is to compute a t-statistic on the slope difference from zero. If the slope is not significant the bioassay is likely to have poor performance; this may be observed as increased variation in the potency results. Another aspect that supports requiring adequate steepness of slope is the use of subset selection algorithms. Without a slope steepness criterion, a subset selection algorithm that seeks to identify subsets of three or more contiguous data points that are straight and parallel might select concentrations on an asymptote. Such subsets are obviously inappropriate to use for potency estimation. How steep or how significant the steepness of the slope should be depends on the assay. This criterion should be set during assay development and possibly refined during assay validation.

4.6 Common Bioassay Models

Most bioassays consist of a series of concentrations or dilutions of both a Test sample and a Standard material. A mathematical model is fit to the concentration–response data, and a relative potency may then be calculated from the parameters of the model. Choice of model may depend on whether quantitative or qualitative data are being analyzed.

For quantitative data, models using parallel response profiles which support comparative evaluation for determining relative potency may provide statistical advantages. If such a model is used, concentrations or dilutions are usually scaled geometrically, e.g., usually in two-fold, log, or half-log increments. If a slope-ratio model is used, concentrations or dilutions can be equally spaced on concentration, rather than log concentration. Several functions may be used for fitting a parallel response model to quantitative data, including a linear function, a higher-order polynomial function, a four-parameter logistic (symmetric sigmoid) function, and a five-parameter logistic function for asymmetric sigmoids. Such functions require a sufficient number of concentrations or dilutions to fit the model. To assess lack of fit of any model it is necessary to have at least one, and preferably several, more concentrations (or dilutions) than the number of parameters that will be estimated in the model. Also, at least one, and better, two, concentrations are commonly used to support each asymptote.

A linear model is sometimes selected because of apparent efficiency and ease of processing. Because bioassay response profiles are usually nonlinear, the laboratory might perform an experiment with a wide range of concentrations in order to identify the approximately linear region of the concentration–response profile. For data that follow a four-parameter logistic model, these are the concentrations near the center of the response region, often from 20% to 80% response when the data are rescaled to the asymptotes. Caution is appropriate in using a linear model because for a variety of reasons the apparently linear region may shift. A stable linear region may be identified after sufficient experience with the assay and with the variety of samples that are expected to be tested in the assay. Data following the four-parameter logistic function may also be linearized by transformation. The lower region of the function is approximately linear when the data are log transformed (log–log fit).

Quantal data are typically fit using more complex mathematical models. A probit or logit model may be used to estimate a percentile of the response curve (usually the 50th percentile) or, more directly, the relative potency of the Test to the Standard. Spearman-Kärber analysis is a non-modeling method that may be employed for determining the 50th percentile of a quantal concentration–response curve.

4.7 Suitability Testing

System suitability and sample suitability assessment should be performed to ensure the quality of bioassay results. System suitability in bioassay, as in other analytical methods, consists of pre-specified criteria by which the validity of an assay (or, perhaps, a run containing several assays) is assessed. Analysts may assess system suitability by determining that some of the parameters of the Standard response are in their usual ranges and that some properties (e.g., residual variation) of the data are in their usual range. To achieve high assay acceptance rates, it is advisable to accept large fractions of these usual ranges (99% or more) and to assess system suitability using only a few uncorrelated Standard response parameters. The choice of system suitability parameters and their ranges may also be informed by empirical or simulation studies that measure the influence of changes in a parameter on potency estimation.

Sample suitability in bioassay is evaluated using pre-specified criteria for the validity of the potency estimate of an individual Test sample, and usually focuses on similarity assessment. System and sample suitability criteria should be established during bioassay development and before bioassay validation. Where there is limited experience with the bioassay, these criteria may be considered provisional.

SYSTEM SUITABILITY

System suitability parameters may be selected based on the design and the statistical model. Regardless of the design and model, however, system suitability parameters should be directly related to the quality of the bioassay. These parameters are generally based on standard and control samples. In parallel-line assays, for example, low values of the Standard slope typically yield estimates of potency with low precision. Rather than reject assays with low slope, analysts may find it more effective to use additional replicate assays until the assay system can be improved to consistently yield higher-precision estimates of poten-

cy. It may be particularly relevant to monitor the range of response levels and location of asymptotes associated with controls or Standard sample to establish appropriate levels of response. A drift or a trend in some of the criteria may indicate the degradation of a critical reagent or Standard material. Statistical process control (SPC) methods should be implemented to detect trends in system suitability parameters.

Two common measures of system suitability are assessment of the adequacy of the model (goodness of fit) and of precision. With replicates in a completely randomized design, a pure error term may be separated from the assessment of lack of fit. Care should be taken in deriving a criterion for lack of fit; the use of the wrong error term may result in an artificial assessment. The lack of fit sum of squares from the model fit to the Standard may, depending on the concentrations used and the way in which the data differ from the model, be a useful measure of model adequacy. A threshold may be established, based on *sensitivity analysis* (assessment of assay sensitivity to changes in the analyte) and/or historical data, beyond which the lack of fit value indicates that the data are not suitable. Note that the Test data are not used here; adequacy of the model for the Test is part of sample suitability.

For assessment of precision, two alternatives may be considered. One approach uses the mean squared error (residual variance) from the model fit to the Standard alone. Because this approach may have few degrees of freedom for the variance estimate, it may be more useful to use a pooled mean squared error from separate model fits to Standard and Test. Once the measure is selected, use historical data and sensitivity analysis to determine a threshold for acceptance.

SAMPLE SUITABILITY

Sample suitability in bioassay generally consists of the assessment of similarity, which can only be done within the assay range. Relative potency may be reported only from samples that both show similarity to Standard, exhibit requisite quality of model fit, and have been diluted to yield an EC_{50} (and potency) within the range of the assay system.

SIMILARITY

In the context of similarity assessment, classical hypothesis (*difference*) testing evaluates a null hypothesis that a measure (a nonsimilarity parameter measuring the difference between Standard and Test concentration–response curves) is zero, with an implicit alternative hypothesis that the measure is non-zero or the statistical assumptions are not satisfied. The usual ("difference test") criterion that the p-value must be larger than a certain critical value in order to declare the sample similar to reference controls the probability that samples are falsely declared nonsimilar; this is the producer's risk of failing good samples. The consumer's risk (the risk that nonsimilar samples are declared similar) is controlled via the precision in the nonsimilarity measure and amount of replication in the assay; typically these are poorly assessed, leaving consumer risk uncontrolled.

In contrast to difference testing, equivalence testing for similarity (assessing whether a 90% confidence interval for a measure of nonsimilarity is contained within specified equivalence bounds) allows only a 5% probability that samples with nonsimilarity measures outside the equivalence boundaries will be declared similar (controlling the consumer's risk). With equivalence testing it is practical to examine and manage the producer's risk by ensuring that there is enough replication in the assay to have good precision in estimating the nonsimilarity measure(s).

For the comparison of slopes, difference tests have traditionally been used to establish parallelism between a Test sample and the Standard sample. Using this approach the laboratory cannot conclude that the slopes are equal. The data may be too variable, or the assay design may be too weak to establish a difference. The laboratory can, however, conclude that the slopes are sufficiently similar using the equivalence testing approach.

Equivalence testing has practical advantages compared to difference testing, including that increased replication yielding improved assay precision will increase the chances that samples will pass the similarity criteria; that decreased assay replication or precision will decrease the chances that samples will pass the similarity criteria; and that sound approaches to combining data from multiple assays of the same sample to better understand whether a sample is truly similar to Standard or not are obtained.

Because of the advantages associated with the use of equivalence testing in the assessment of similarity, analysts may transition existing assays to equivalence testing or may implement equivalence testing methods when changes are made to existing assays. In this effort, it is informative to examine the risk that the assay will fail good samples. This risk depends on the precision of the assay system, the replication strategy in the assay system, and the critical values of the similarity parameters (this constitutes a *process capability analysis*). One approach to transitioning an established assay from difference testing to equivalence testing (for similarity) is to use the process capability of the assay to set critical values for similarity parameters. This approach is reasonable for an established assay because the risks (of falsely declaring samples similar and falsely declaring samples nonsimilar) are implicitly acceptable, given the assay's history of successful use.

Similarity measures may be based on the parameters of the concentration–response curve and may include the slope for a straight parallel-line assay; intercept for a slope-ratio assay; the slope and asymptotes for a four-parameter logistic parallel-line assay; or the slope, asymptotes, and nonsymmetry parameter in a five-parameter sigmoid model. In some cases, these similarity measures have interpretable, practical meaning in the assay; certain changes in curve shape, for example, may be associated with specific changes (e.g., the presence of a specific active contaminant) in the product. When possible, discussion of these changes and their likely effects is a valuable part of setting appropriate equivalence boundaries.

IMPLEMENTING EQUIVALENCE TESTING FOR SIMILARITY (PARALLELISM)

As previously stated, many statistical procedures for assessing similarity are based on a null hypothesis stating that similarity is present and the alternative hypothesis of there being a state of nonsimilarity. Failure to find that similarity is statistically improbable is then taken as a conclusion of similarity. In fact, however, this failure to establish a probabilistic basis for nonsimilarity does not prove similarity. Equivalence testing provides a method for the analyst to proceed to a conclusion (if warranted by the data) of *sufficiently similar* while controlling the risk of doing so inappropriately. The following provides a sequence for this process of implementing equivalence testing.

Step 1: Choose a measure of nonsimilarity.

For the parallel-line case, this could be the difference or ratio of slopes. (The ratio of slopes can be less sensitive to the value of the slope. Framing the slope difference as a proportional change from Standard rather than in absolute slope units has an advantage because it is invariant to the units on the concentration and response axes.) For a slope-ratio assay, the measure of nonsimilarity can be the difference in y-intercepts between Test and Standard samples. Again, it can be advantageous to frame this difference as a proportion of the (possibly transformed) response range of Standard to make the measure invariant to the units of the response.

The determination of similarity could be based on the individual parameters, one at a time; for the four-parameter logistic model, similarity between Standard and Test samples can be assessed discretely for the upper asymptote, the slope, and the lower asymptote. If sigmoid curves with additional parameters are used to fit bioassay data, it is also important to consider addressing similarity between Standard and Test preparations of the additional curve parameters (e.g., asymmetry parameter of the five-parameter model). Alternatively, evaluation of similarity can be based on a single composite measure of nonparallelism, such as the *parallelism sum of squares*. This is found as the difference in residual sum of squared errors (RSSE) between the value obtained from fitting the Standard and Test curves separately and the value obtained from imposing parallelism:

$$\text{Parallelism sum of squares} = RSSE_p - RSSE_s - RSSE_t$$

where the subscripts P, S, and T denote Parallel model, Standard model, and Test model, respectively. With any composite measure, the analyst must consider the implicit relative weighting of the importance of the three (or more) curve regions and whether the weighting is appropriate for the problem at hand. For the parallelism sum of squares, for example, with nonlinear models, the weighting given to the comparison of the asymptotes depends on the amount of data in the current assay on and near the asymptotes.

Step 2: Specify a range of acceptable values, typically termed an equivalence interval or "indifference zone," for the measure of nonsimilarity.

The challenge in implementing equivalence testing is in setting appropriate equivalence bounds for the nonsimilarity measures. Ideally, information is available to link variation in similarity measures to meaningful differences in biological function (as measured by the bioassay). Information may be available from evaluation of orthogonal assays. The following four approaches can be used to determine this interval. If pharmacopeial limits have been specified for a defined measure of nonsimilarity, then the assay should satisfy those requirements.

a. The first approach is to compile historical data that compare the Standard to itself and using these data to determine the equivalence interval as a tolerance interval for the measure of nonparallelism. The advantage of using historical data is that they give the laboratory control of the false failure rate (the rate of failing a sample that is in fact acceptable). The disadvantage is that there is no control of the false pass rate (the rate of passing a sample that may have an unacceptable difference in performance relative to the Standard). The equivalence interval specification developed in this way is based solely on assay capability. Laboratories that use this approach should take caution that an imprecise assay in need of improvement may yield such a wide equivalence interval that no useful discrimination of nonsimilarity is possible.

b. Approach (a) is simple to implement in routine use and can be used with assay designs that do not provide reliable estimates of within-assay variation and hence confidence intervals. However, there is a risk that assays with larger than usual amounts of within-assay variation can pass inappropriately. The preferable alternative to (a) is therefore to determine a tolerance interval for the confidence interval for the measure of nonparallelism. The following is particularly appropriate to transition an existing assay with a substantial body of historical data on both Standard and Test samples from a difference testing approach to an equivalence approach:

i. For each value of the measure of nonparallelism from the historical data, determine a 95% confidence interval, (m,n).

ii. For each confidence interval, determine its maximum departure from perfect parallelism. This is max(|m|, |n|) for differences, max(1/m,n) for ratios, and simply n for quantities that must be positive, such as a sum of squares.

iii. Determine a tolerance interval for the maximum departures obtained in (ii). This will be a one-sided tolerance interval for these necessarily positive quantities. A nonparametric tolerance interval approach is preferred.

iv. "Sufficiently parallel" is concluded for new data if the confidence interval for the measure of nonparallelism falls completely within the interval determined in (iii).

Approaches (a) and (b), through their reliance on assay capability, control only the false fail rate, and neglect the false pass rate. Incorporating information from sources other than the evaluation of assay capability provides control of the false pass rate. Approaches (c) and (d) are means to this end.

c. The third approach starts with historical data comparing the Standard to itself and adds data comparing the Standard to known failures, e.g., to degraded samples. Compare values of the measure of nonsimilarity for data for which a conclusion of similarity is appropriate (Standard against itself) and data for which a conclusion of similarity is not appropriate, e.g., degraded samples. Based on this comparison, determine a value of the measure of nonsimilarity that discriminates between the two cases. If this approach is employed, a range of samples for which a conclusion of similarity is not appropriate should be utilized, including samples with the minimal important nonsimilarity. For nonlinear models, this comparison also can be used to determine which parameters should be assessed; some may not be sensitive to the failures that can occur with the specific assay or collection of nonsimilar samples.

d. The fourth approach is based on combining a sensitivity analysis of the assay curve to nonsimilarity parameters with what is known about the product and the assay. It is particularly helpful if information is available that links a shift in one or more nonsimilarity measures to properties of the product. These measures may be direct (e.g., conformational changes in a protein) or indirect (e.g., changes in efficacy or safety in an animal model). A complementary approach is provided by a limited sensitivity analysis that combines analyst and biologist judgment regarding the magnitude of shifts in a nonsimilarity parameter that are meaningful, with simulation and/or laboratory experiments, to demonstrate thresholds for similarity parameters that provide protection against important nonsimilarity. Additionally, risk analysis may be informed by the therapeutic index of the drug.

Step 3. Examine whether the value of the nonsimilarity measure is found within the equivalence interval of acceptable values. For approaches (a) and (b), compare the obtained value of the measure of nonparallelism (a) or its confidence interval (b) to the interval obtained at the beginning of Step 2. The value must be within the limits if one uses (a), or the confidence interval must be completely within the limits if one uses (b).

An alternative to the approach described above [for (a)] is to use an average (historical) value for the variance of the ratio or difference in a similarity parameter—obtained from some number of individual assays—to compute an acceptance interval for a point estimate of the similarity parameter. This approach is simpler to implement in routine use and can be used with assay designs that are unable to provide reliable estimates of within-assay variation. However, there is a price. The equivalence testing approach that relies on assay-specific (within-assay) measure(s) of variation (i.e., the confidence intervals) is conservative in the sense that it will fail to pass similarity for samples from assays that have larger than usual amounts of within-assay variation. Using an acceptance region for a similarity parameter—rather than an acceptance region for confidence intervals for the similarity parameter—loses this conservative property and hence is not preferred where alternatives exist.

For approach (c), an approach that essentially treats the parallelism as a discrimination problem may be used. The choice of the cut point in (c) should take into account the rates of false positive and false negative decisions (and the acceptable risks to the laboratory) and should reflect the between-assay variability in precision. Thus it is reasonable to compare the point estimate of the measure of nonparallelism to the cut point and to not use confidence intervals. This approach is simpler to implement in routine use and can be used with assay designs that cannot provide reliable estimates of within-assay variation.

For approach (d), demonstrate that the measure of nonsimilarity is significantly greater than the lower endpoint of the acceptance interval and significantly less than the upper endpoint. (If the acceptance interval is one-sided, then apply only the single applicable test.) This is use of the TOST approach. For most situations, TOST can be most simply implemented by calculating a 90% two-sided confidence interval, which corresponds to a 5% equivalence test. If this confidence interval lies entirely within the equivalence interval specified at the beginning of Step 2, then similarity is sufficiently demonstrated. For parallel-line models, one can use either (1) a confidence interval based on the value of the difference of the slopes ±k times the standard error of that value, or (2) Fieller's Theorem for the ratio of slopes may be used. For slope ratio models use the confidence interval for the difference of intercepts. For nonlinear models, there is evidence that these simple confidence interval methods do not attain the stated level of confidence, and methods based on likelihood profile or resampling are more appropriate.

RANGE

The range for a relative potency bioassay is the interval between the upper and lower relative potencies for which the bioassay is shown to have suitable levels of precision, relative accuracy, linearity of log potency, and success rates for system and sample suitability. It is straightforward to determine whether or not a sample that is similar to a Standard has a relative potency within the (validated) range of the assay system. For samples that are not similar according to established criteria, it is more challenging to determine whether a relative potency estimate for the sample might be obtained. In a nonlinear parallel-line assay a sample that does not have data on one asymptote might be assumed to be out of the potency range of the assay. In a parallel straight-line assay a sample that does not have three or more points on the steep portion of the response curve may be out of the potency range of the assay. For samples that have not been shown to be similar to reference it is not appropriate to report potency or to construct a ratio of EC_{50}s from unrestricted fits. As such samples may be out of the assay range, it may be useful to shift the dilution of the test sample for a subsequent assay on the basis of an estimate of relative activity. This estima-

General Chapters

ted relative activity may be obtained via the ratio of the concentrations of Standard and Test that yields responses that match the reference response at the reference EC_{50}.

4.8 Outliers

Bioassay data should be screened for outliers before relative potency analysis. Outliers may be simple random events or a signal of a systematic problem in the bioassay. Systematic error that generates outliers may be due to a dilution error at one or more concentrations of a Test sample or the Standard or due to a mechanical error (e.g., system malfunction). Several approaches for outlier detection can be considered. Visual inspection is frequently utilized but should be augmented with a more objective approach to avoid potential bias.

An outlier is a datum that appears not to belong among the other data present. An outlier may have a distinct, identifiable cause, such as a mistake in the bench work, equipment malfunction, or a data recording error, or it could just be an unusual value relative to the variability typically seen and may appear without an identifiable cause. The essential question pertaining to an outlier becomes: Is the apparent outlier sampled from the same population as the other, less discordant, data, or is it from another population? If it comes from the same population and the datum is, therefore, an unusual (yet still legitimate) value obtained by chance, then the datum should stand. If it comes from another population and the datum's excursive value is due to human error or instrument malfunction, then the datum should be omitted from calculations. In practice, the answer to this essential question is often unknown, and investigations into causes are often inconclusive. Outlier management relies on procedures and practices to yield the best answer possible to that essential question and to guide response accordingly.

General chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses outlier labeling, identification, and rejection; statistical methods are included. General chapter ⟨1010⟩ also lists additional sources of information that can provide a comprehensive review of the relevant statistical methodology. General chapter ⟨1010⟩ makes no explicit remarks regarding outlier analysis in linear or nonlinear regression. Outlier analysis techniques appropriate for data obtained from regression of response on concentration can be used. Some remarks about outliers are provided here in the context of bioassays to emphasize or complement the information in ⟨1010⟩.

Of the procedures employed for analysis of drug compounds and biological drugs, the bioassay may be expected to be the most prone to outlying data. The management of outliers is appropriate with bioassay data on at least two levels: where an individual datum or a group of data (e.g., data at a concentration) can be checked against expected responses for the sample and concentration; and, separately, when estimates of relative potency from an assay can be checked for consistency with other independent estimates of the potency of the same material.

Three important aspects of outlier management are prevention, labeling, and identification.

Outlier prevention is preferred for obvious reasons, and is facilitated by procedures that are less subject to error and by checks that are sensitive to the sorts of errors that, given the experience gained in assay development, may be expected to occur. In effect, the error never becomes an outlier because it is prevented from occurring.

Good practice calls for the examination of data for outliers and labeling ("flagging") of the apparently outlying observation(s) for investigation. If investigation finds a cause, then the outlying datum may be excluded from analysis. Because of the ordinary occurrence of substantial variability in bioassay response, a laboratory's investigation into the outlying observation is likely to yield no determinable cause. However, the lack of evidence regarding an outlier's cause is not a clear indication that statistical outlier testing is warranted. Knowledge of the typical range of assay response variability should be the justification for the use of statistical outlier tests.

Outlier identification is the use of rules to confirm that the values are inconsistent with the known or assumed statistical model. For outliers with no determined cause, it is tempting to use statistical outlier identification procedures to discard unusual values. Discarding data solely because of statistical considerations should be a rare event. Falsely discarding data leads to overly optimistic estimates of variability and can bias potency estimates. The laboratory should monitor the failure rate for its outlier procedure and should take action when this is significantly higher than expected.

Statistical procedures for outlier identification depend on assumptions about the distribution of the data without outliers. Identification of data as outliers may mean only that the assumption about distribution is not correct. If dropping outliers because of statistical considerations is common, particularly if outliers tend to occur more often at high values or at high responses, then this may be an indication that the data require some adjustment, such as log transformation, as part of the assay procedure. Two approaches to statistical assessment of outlying data are replication-based and model-based.

REPLICATION-BASED APPROACHES

When replicates are performed at concentrations of a Test sample and the Standard, an "extra variability" (EV) criterion may be employed to detect outliers. Historical data can be analyzed to determine the range in variability commonly observed among replicates, and this distribution of ranges can be used to establish an extreme in the range that might signal an outlier. Metrics that can be utilized are the simple range (maximum replicate minus minimum replicate), the standard deviation, or the CV or RSD among replicates. However, if the bioassay exhibits heterogeneity of variability, assumptions about uniform scatter of data are unsupported. Analysts can use a variable criterion across levels in the bioassay, or they can perform a transformation of the data to a scale that yields homogeneity of variability. Transformation can be performed with a POM approach

General Chapters

as discussed previously. Where heterogeneity exists nonnormality is likely present, and the range rather than standard deviation or RSD should be used.

The actions taken upon detection of a potential outlier depend in part on the number of replicates. If EV is detected within a pair (n = 2) at a concentration of a Test sample or the Standard, it will not always be clear which of the replicates is aberrant, and the laboratory should eliminate the concentration from further processing. If more than two replicates are performed at each dilution the laboratory may choose to adopt a strategy that identifies which of the extremes may be the outlier. Alternatively, the laboratory may choose to eliminate the dilution from further processing.

MODEL-BASED APPROACHES

Model-based approaches may be used to detect outliers within bioassay data. These approaches use the residuals from the fit of an appropriate model. In general, if using model-based methods to identify potential outliers, the models used may make fewer assumptions about the data than the models used to assess suitability and estimate potency. For example, a non-parametric regression (smoothing) model may be useful.

Lastly, an alternative to discarding outlying data is to use robust methods that are less sensitive to influence by outlying observations. Use of the median rather than the mean to describe the data's center exemplifies a robust perspective. Also, regression using the method of least squares, which underlies many of the methods in this chapter, is not robust in the presence of outliers. The use of methods such as robust regression may be appropriate but is not covered in the USP bioassay chapters.

4.9 Fixed and Random Effects in Models of Bioassay Response

The choice of treating factors as fixed or random is important for the bioassay design, the development experiments, the statistical analysis of data, and the bioassay validation. Fixed effects are factors for which all levels, or all levels of interest, are discretely present, like sample, concentration, temperature and duration of thaw, and incubation time. Data for a response at some level, or combination of levels, of a fixed factor, can predict future responses. Fixed effects are expected to cause a consistent shift in responses. Analysts study fixed effects by controlling them in the design and examining changes in means across levels of the factor.

Random effects are factors of which the levels in a particular run of an assay are considered representative of levels that could be present. That is, there is no expectation that any specific value of the random factor will influence response. Rather, that value may vary subject to some expected distribution of values and thus may be a source of variability. For example, there is no desire to predict assay response for a specific day, but there is interest in predicting the variation in response associated with the factor "day". Examples of random effects include reagent lot, operator, or day if there is no interest in *specific* reagent lots, operators, or day as sources of variability. Analysts may study random effects by measuring the variance components corresponding to each random effect. Variance components can be estimated well only if there are an appreciable number of levels of each random effect. If there are, for example, only two or three reagent lots or analysts present, the variation associated with these factors will be poorly estimated.

Making a correct choice regarding treating a factor as fixed or random is important to the design of the assay and to proper reporting of its precision. Treating all factors as fixed, for example, leads to an understatement of assay variability because it ignores all sources of variability other than replication. The goal is to identify specific sources of variability that can be controlled, to properly include those factors in the design, and then to include other factors as random.

If the factor may switch from random to fixed effect or vice versa, the factor should normally be modeled as a random effect. For example, reagent lots cannot be controlled, so different lots are typically considered to cause variability, and reagent lot would be considered a random effect. However, if a large shift in response values has been traced to a particular lot, a comparison among a set of lots could be performed using reagent lot as a fixed effect. Similarly, within-assay location (e.g., block, plate, plate row, plate column, or well) or sequence may be considered a source of random variation or a source of a consistent (fixed) effect.

Assay designs that consist of multiple factors are efficient, but require corresponding statistical techniques that incorporate the factors as fixed or random effects in the analysis. If all factors are fixed, the statistical model is termed a fixed-effects model. If all are random, it is termed a random-effects model. If some factors are fixed and some random, the model is a mixed-effects model. Note that the concepts of fixed and random effects apply to models for quantitative, qualitative and integer responses. For assay designs that include multiple experimental units (e.g., samples assigned to sets of tubes and concentrations assigned to pre-plate tubes) a mixed-effects model in which the experimental units are treated as random effects is particularly effective. Additional complexity is added by the presence of designs with crossed random effects (e.g., each operator used material from one or more reagent batches, but many reagent batches were used by multiple operators). This can cause methodological and computational challenges for model fitting, especially when the designs are unbalanced.

5. STAGES IN THE BIOASSAY DEVELOPMENT PROCESS

Given the ubiquity of cell-based assays and the motivation to use one bioassay system to provide context for discussion, the development of a cell-based bioassay will be used to illustrate the stages in the bioassay development continuum.

5.1 Design: Assay Layout, Blocking, and Randomization

Most cell-based assays are performed using a cell culture plate (6-, 12-, 96-, or 384-well micro titer plate). Ideally, a plate is able to provide a uniform substrate for experimental treatments in all wells, including after wash steps and incubations. However, regardless of assay conditions intended to minimize the potential for bias (e.g., good analyst technique, careful calibration of pipets, controlled incubation time, and temperature), systematic gradients on the plate, independent of experimental treatments, may be observed. These gradients may occur across rows, across columns, or from the edge to the center of the plate and are often called *plate effects.* Even moderate or inconsistent plate effects should be addressed during assay development, by means of plate layout strategies, blocking, randomization, and replication.

Plate effects can be evaluated in a *uniformity trial* in which a single experimental treatment, such as an assay concentration chosen from the middle section of the concentration–response curve, is used in all wells of the plate. *Figure 1* provides an example of what may be observed; a trend of decreasing signal is evident from right to left. In this case, it was discovered that the plate washer was washing more briskly on the left side of the plate, and required adjustment to provide uniform washing intensity and eliminate the gradient. Another common plate effect is a differential cell-growth pattern in which the outer wells of the plate grow cells in such a way that the assay signal is attenuated. This is such a persistent problem that the choice is often made to not use the outer wells of the assay plate. Because location effects are so common, designs that place replicates (e.g., of sample by concentration combinations) in adjacent wells should be avoided.

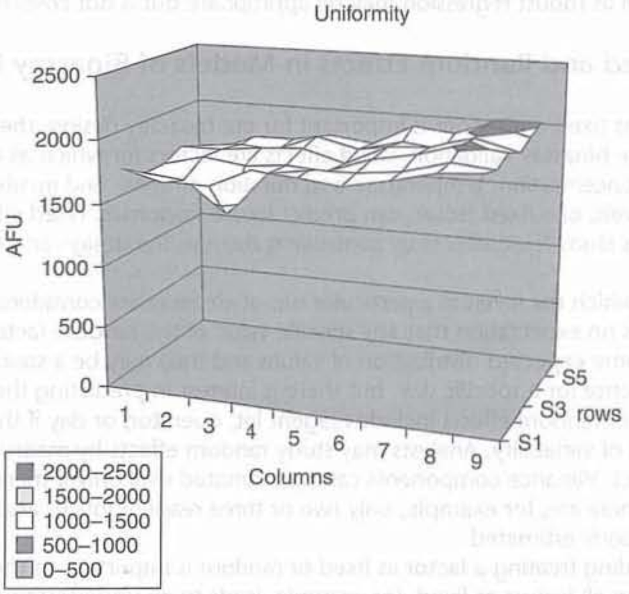


Figure 1. Plot of change in assay response across a plate.

Blocking is the grouping of related experimental units in experimental designs. Blocks may consist of individual 96-well plates, sections of 96-well plates, or 96-well plates grouped by analyst, day, or batch of cells. The goal is to isolate any systematic effects so that they do not obscure the effects of interest. A *complete block design* occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) are applied to experimental units for that factor within a single block. An *incomplete block design* occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

Randomization is a process of assignment of treatment to experimental units based on chance so that all such experimental units have an equal chance of receiving a given treatment. Although challenging in practice, randomization of experimental treatments has been advocated as the best approach to minimizing assay bias or, more accurately, to protecting the assay results from known and unknown sources of bias by converting bias into variance. While randomization of samples and concentrations to individual plate wells may not be practical, a plate layout can be designed to minimize plate effects by alternating sample positions across plates and the pattern of dilutions within and across plates. Where multiple plates are required in an assay, the plate layout design should, at a minimum, alternate sample positions across plates within an assay run to accommodate possible bias introduced by the analyst or equipment on a given day. It is prudent to use a balanced rotation of layouts on plates so that the collection of replicates (each of which uses a different layout) provides some protection against likely sources of bias.

Figure 2 illustrates a patterned assay design that lacks randomization and is susceptible to bias. Dilutions and replicates of the Test preparations (A and B) and the Standard (R) are placed together sequentially on the plate. Bias due to a plate or incubator effect can influence some or all of the concentrations of one of the samples. Note that in *Figures 2* through *5* all outer plate wells are left as blanks to protect against *edge effect.*

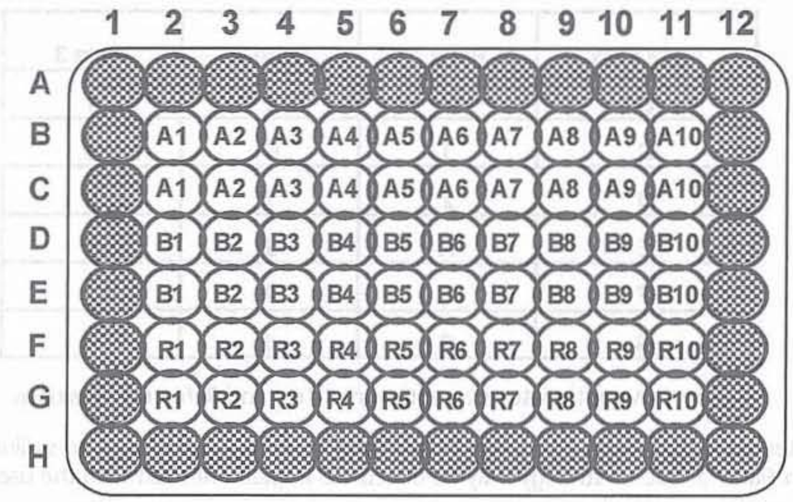


Figure 2. A highly patterned plate.

A layout that provides some protection from plate effects and can be performed manually is a *strip-plot design,* shown in *Figure 3.* Here samples are randomized to rows of a plate and dilution series are performed in different directions in different sections (blocks) on the plate to mitigate bias across columns of the plate. An added advantage of the strip-plot design is the ability to detect location effects by the interaction of sample and dilution direction (left-to-right or right-to-left).

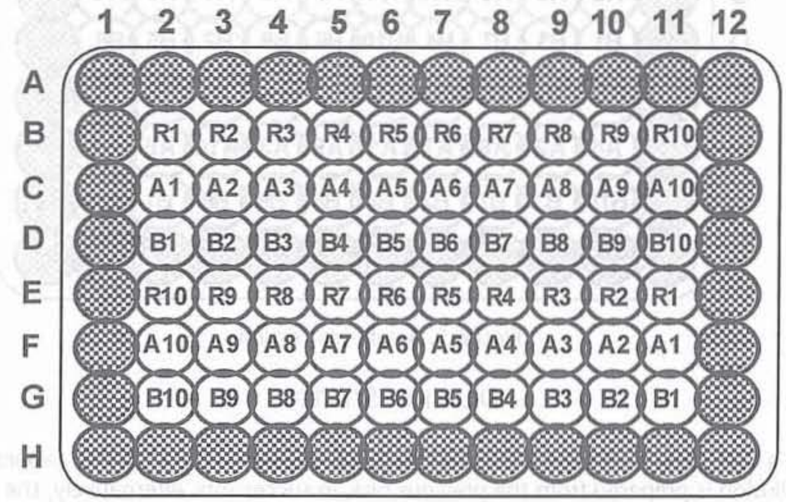


Figure 3. A strip-plot design.

Figure 4 illustrates an alternation of Test (Test sample 1 = "1"; Test sample 2 = "2") and Standard ("R") positions on multiple plates, within a single assay run; this protects against plate row effects. Combining the two methods illustrated if *Figures 3* and *4* can effectively help convert plate bias into assay variance. Assay variance may then be addressed, as necessary, by increased assay replication (increased number of plates in an assay).

Plate Row	Plate 1	Plate 2	Plate 3
B	R	2	1
C	1	R	2
D	2	1	R
E	R	2	1
F	1	R	2
G	2	1	R

Figure 4. A multi-plate assay with varied Test and Reference positions.

A *split-plot design,* an alternative that assigns samples to plate rows randomly and randomizes dilutions (concentrations) within each row, is seen in *Figure 5*. Such a strategy may be difficult to implement even with the use of robotics.

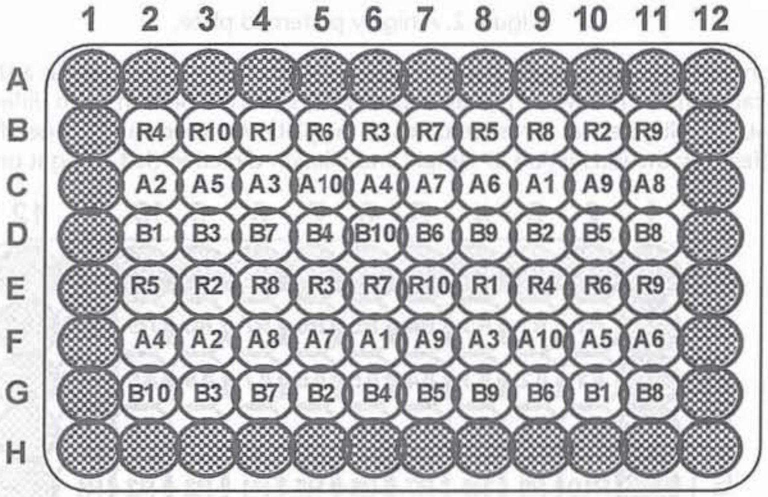


Figure 5. A split-plot design.

Dilution Strategy

Assay concentrations of a Test sample and the Standard can be obtained in different ways. Laboratories often perform serial dilutions, in which each dilution is prepared from the previous one, in succession. Alternatively, the laboratory may prepare wholly independent dilutions from the Test sample and Standard to obtain independent concentration series. These two strategies result in the same nominal concentrations, but they have different properties related to error. Serial dilutions are subject to propagation of error across the dilution series, and a dilution error made at an early dilution will result in correlated, non-independent observations. Correlations may also be introduced by use of multichannel pipets. Independent dilutions help mitigate the bias resulting from dilution errors.

It is noteworthy that when working to improve precision, the biggest reductions in variance come when replicating at the highest possible levels of nested random effects. This is particularly effective when these highest levels are sources of variability. To illustrate: replicating extensively within a day for an assay known to have great day-to-day variation is not effective in improving precision of reportable values.

5.2 Development

A goal of bioassay development is to achieve optimal bioassay relative accuracy and precision of the potency estimate. An endpoint of assay development is the completed development of the assay procedure, a protocol for the performance of the bioassay. The procedure should include enough detail so that a qualified laboratory with a trained analyst can perform the procedure in a routine manner. A strategic part of development is a look forward toward performance maintenance. Standard operating procedures for reagent and technician qualification, as well as for calibration of the working Standard, help complete the bioassay development package.

ONE FACTOR AT A TIME VERSUS DESIGN OF EXPERIMENTS

Bioassay development proceeds through a series of experiments in which conditions and levels of assay factors are varied to identify those that support a reliable and robust bioassay qualified for routine use. Those experiments may be conducted one factor at a time (OFAT), studying each parameter separately to identify ideal conditions, or through the use of multi-factor design of experiments (DOE). DOE is an efficient and effective strategy for developing a bioassay and improving bioassay performance, thus helping to obtain a measurement system that meets its requirements. In comparison to OFAT, DOE generally requires fewer experiments and also provides insight into interactions of factors that affect bioassay performance. Assay development using DOE may proceed through a series of steps: process mapping and risk analysis; screening; response optimization; and confirmation.

PROCESS MAPPING AND RISK ANALYSIS

Bioassay optimization may begin with a systematic examination and risk assessment to identify those factors that may influence bioassay response. It is useful to visualize bioassay factors using a bioassay process map such as a cause-and-effect or fishbone diagram. Using the process map as a guide, the laboratory can examine assay factors that might affect assay performance, such as buffer pH, incubation temperature, and incubation time. Historical experience with one or several of the bioassay steps, along with sound scientific judgment, can identify key factors that require further evaluation. One tool that may be used to prioritize factors is a failure mode and effects analysis. Factors are typically scored by the combination of their potential to influence assay response and the likelihood that they will occur. The laboratory must be careful to recognize potential interactions between assay factors.

SCREENING

Once potential key factors have been identified from process mapping and risk analysis, the laboratory may conduct an initial screening experiment to probe for effects that may require control. Screening designs such as factorial and fractional factorial designs are commonly used for this purpose. Software is available to assist the practitioner in the selection of the design and in subsequent analysis. Analysts should take care, however, to understand their assumptions about design selection and analysis to ensure accurate identification of experimental factors.

RESPONSE OPTIMIZATION

A screening design will usually detect a few important factors from among those studied. Such factors can be further studied in a response-optimization design. Response-optimization designs such as central composite designs are performed to determine optimal settings for combinations of bioassay factors for achieving desired response. The information obtained from response optimization may be depicted as a response surface and can be used to establish ranges that yield acceptable assay performance and will be incorporated into the bioassay procedure.

In the parlance of Quality by Design (QbD), the "region" where the combined levels of input variables and process parameters have been demonstrated to provide acceptable assay performance is described as the *design space* for the bioassay. Establishing a true design space for a bioassay is challenging; some but not all factors and levels of random factors will be included in the development DOE, and there is no assurance that the design space is not sensitive to unstudied random factors. Similarly, there is little assurance that the assay (design space) is robust to random factors that are studied using small samples (or non-random samples of levels). Elements of DOE that may be considered include the use of blocks; deliberate confounding among interactions that are of lower interest, or known to be unimportant; robust design (response surface designs with random effects); and use of split-plot, strip-plot, or split-lot designs.

CONFIRMATION

The mathematical model depicting assay performance as a function of changes in key assay factors is an approximation; thus, it is customary to confirm performance at the ideal settings of the bioassay. Confirmation can take the form of a qualification trial in which the assay is performed, preferably multiple independent times using optimal values for factors. Alternatively, the laboratory may determine that the bioassay has been adequately developed and may move to validation. Qualification is a good practice, not a regulatory requirement. The decision to perform confirmatory qualifying runs or to proceed to validation depends upon the strength of the accumulated information obtained throughout development.

5.3 Data Analysis during Assay Development

Analysis of bioassay data during assay development enables analysts to make decisions regarding the statistical model that will be used for routine analysis, including transformation and/or weighting of data, and the development of system and sample suitability criteria. The analysis also provides information regarding which elements of design structure should be used during outlier detection and the fitting of a full model. This may also include a plan for choosing subsets of data, such as a linear portion, for analysis or, for nonlinear bioassays, a model reduction strategy for samples similar to Standard. Once these decisions are made and proven sound during validation, they don't need to be reassessed with each performance of the assay. A process approach to enabling these decisions follows.

Step 1: Choose an appropriate statistical model (also see section 4.6 Common Bioassay Models).

Given the complexity of bioassays and the motivation to use an approach proven reliable, fairly standardized analytical models are common in the field of bioassay analysis. Nonetheless, many considerations are involved in choosing the most appropriate statistical model. First, the model should be appropriate for the type of assay endpoint—continuous, count, or dichotomous. Second, the model should incorporate the structure of the assay design. For any design other than completely randomized, there will be terms in the model for the structural elements. These could be, for example, within-plate blocking, location of cage in the animal facility, day, etc. A third consideration, applicable to continuous endpoints, involves whether to use a regression model or a means model (an analysis of variance model that fits a separate mean at each dilution level of each sample tested), with appropriate error terms. A means model can be appropriate at this stage because it makes no assumptions about the shape of the concentration–response curve.

Step 2: Fit the chosen statistical model to the data without the assumption of parallelism, and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance.

Transform the data as necessary or, if needed, choose a weighting scheme (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation*). Use as large a body of assay data, from independent assays, as possible. The primary goal is to address any departure from normality and from constant variance of responses across the range of concentrations in the assay. Step 2 will likely alternate between imposing a transformation and assessing the distribution of the residuals.

Step 3: Screen for outliers, and remove as is appropriate.

This step normally follows the initial choice of a suitable transformation and/or weighting method. Ideally the model used for outlier detection contains the important elements of the assay design structure, allows nonsimilar curves, and makes fewer assumptions about the functional shape of the concentration–response curve than did the model used to assess similarity. See section 4.8 *Outliers* and general chapter ⟨1010⟩ for discussion of outlier detection and removal. In some cases, outliers may be so severe that a reasonable model cannot be fit, and thus residuals will not be available. In such cases, it is necessary to screen the raw data for outliers before attempting to fit the model.

During assay development, a strategy should be developed for the investigation and treatment of an outlier observation, including any limits on how many outliers are acceptable. Include these instructions in the assay SOP. Good practice includes recording the process of an investigation, outlier test(s) applied, and results therefrom. Note that outlier procedures must be considered apart from the investigation and treatment of an out-of-specification (OOS) result (reportable value). Decisions to remove an outlier from data analysis should not be made on the basis of how the reportable value will be affected (e.g., a potential OOS result). Removing data as outliers should be rare. If many values from a run are removed as outliers, that run should be considered suspect.

Step 4: Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.

Step 5: If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear (see section 4.5 Linearity of Concentration–Response Data).

Step 6: Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.

5.4 Bioassay Validation

The bioassay validation is a protocol-driven study that demonstrates that the procedure is fit for use. A stage-wise approach to validation may be considered, as in a "suitable for intended use" validation to support release of clinical trial material, and a final, comprehensive validation prior to BLA or MAA filing. Preliminary system and sample suitability controls should be established and clearly described in the assay procedure; these may be finalized based on additional experience gained in the validation exercise. Chapter ⟨1033⟩ provides validation comprehensive discussion of bioassay validation.

5.5 Bioassay Maintenance

The development and validation of a bioassay, though discrete operations, lead to ongoing activities. Assay improvements may be implemented as technologies change, as the laboratory becomes more skilled with the procedure, and as changes to bioassay methodology require re-evaluation of bioassay performance. Some of these changes may be responses to unexpected performance during routine processing. Corrective action should be monitored using routine control procedures. Substantial changes may require a study verifying that the bioassay remains fit for use. An equivalence testing approach can be used to

show that the change has resulted in acceptable performance. A statistically-oriented study can be performed to demonstrate that the change does not compromise the previously acceptable performance characteristics of the assay.

ASSAY TRANSFER

Assay transfer assumes both a known intended use of the bioassay in the recipient lab and the associated required capability for the assay system. These implicitly, though perhaps not precisely, demarcate the limits on the amount of bias and loss of precision allowed between labs. Using two laboratories interchangeably to support one product will require considering the variation between labs in addition to intermediate precision for sample size requirements to determine process capability. For a discussion and example pertaining to the interrelationship of bias, process capability, and validation, see *A Bioassay Validation Example* in ⟨1033⟩.

IMPROVING OR UPDATING A BIOASSAY SYSTEM

A new version of a bioassay may improve the quality of bias, precision, range, robustness, specificity, lower the operating costs or offer other compelling advantages. When improving or updating a bioassay system a bridging study may be used to compare the performance of the new to the established assay. A wide variety of samples (e.g., lot release, stability, stressed, critical isoforms) can be used for demonstrating equivalence of estimated potencies. Even though the assay systems may be quite different (e.g., an animal bioassay versus a cell-based bioassay), if the assays use the same Standard and mechanism of action, comparable potencies may reasonably be expected. If the new assay uses a different Standard, the minimum requirement for an acceptable comparison is a unit slope of the log linear relationship between the estimated potencies. An important implication of this recommendation is that poor precision or biased assays used early can have lasting impact on the replication requirements, even if the assay is later replaced by an improved assay.

⟨1033⟩ BIOLOGICAL ASSAY VALIDATION

1. INTRODUCTION

Biological assays (also called bioassays) are an integral part of the quality assessment required for the manufacturing and marketing of many biological and some non-biological drug products. Bioassays commonly used for drug potency estimation can be distinguished from chemical tests by their reliance on a biological substrate (e.g., animals, living cells, or functional complexes of target receptors). Because of multiple operational and biological factors arising from this reliance on biology, they typically exhibit a greater variability than do chemically-based tests.

Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes of a biological drug product. As described in the ICH Guideline entitled Specifications: Test Procedures And Acceptance Criteria For Biotechnological/Biological Products (Q6B), section 2.1.2, bioassay techniques may measure an organism's biological response to the product; a biochemical or physiological response at the cellular level; enzymatic reaction rates or biological responses induced by immunological interactions; or ligand- and receptor-binding. As new biological drug products and new technologies emerge, the scope of bioassay approaches is likely to expand. Therefore, general chapter *Biological Assay Validation* ⟨1033⟩ emphasizes validation approaches that provide flexibility to adopt new bioassay methods, new biological drug products, or both in conjunction for the assessment of drug potency.

Good manufacturing practice requires that test methods used for assessing compliance of pharmaceutical products with quality requirements should meet appropriate standards for accuracy and reliability. Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. *USP* general chapter *Validation of Compendial Procedures* ⟨1225⟩ and ICH Q2(R1) describe the assay performance characteristics (parameters) that should be evaluated for procedures supporting small-molecule pharmaceuticals. Although evaluation of these validation parameters is straightforward for many types of analytical procedures for well-characterized, chemically-based drug products, their interpretation and applicability for some types of bioassays has not been clearly delineated. This chapter addresses bioassay validation from the point of view of the measurement of activity rather than mass or other physicochemical measurements, with the purpose of aligning bioassay performance characteristics with uses of bioassays in practice.

Assessment of bioassay performance is a continuous process, but bioassay validation should be performed when development has been completed. Bioassay validation is guided by a validation protocol describing the goals and design of the validation study. General chapter ⟨1033⟩ provides validation goals pertaining to *relative potency* bioassays. Relative potency bioassays are based on a comparison of bioassay responses for a Test sample to those of a designated Standard that provides a quantitative measure of the Test bioactivity relative to that of the Standard.

Validation parameters discussed include *relative accuracy, specificity, intermediate precision,* and *range.* Laboratories may use *dilutional linearity* to verify the *relative accuracy* and *range* of the method. Although robustness is not a requirement for validation, general chapter ⟨1033⟩ recommends that a bioassay's robustness be assessed prior to validation. In addition, ⟨1033⟩ describes approaches for validation design (sample selection and replication strategy), validation acceptance criteria, data analysis and interpretation, and finally bioassay performance monitoring through quality control. Documentation of bioassay validation results is also discussed, with reference to pre-validation experiments performed to optimize bioassay performance. In the remainder of general chapter ⟨1033⟩ the term "bioassay" should be interpreted as meaning "relative potency bioassay".

2. FUNDAMENTALS OF BIOASSAY VALIDATION

The goal of bioassay validation is to confirm that the operating characteristics of the procedure are such that the procedure is suitable for its intended use. The issues involved in developing a bioassay are described in greater detail in general chapter ⟨1032⟩ and are assumed resolved by the time the bioassay is in validation. Included in those decisions will be identification of what constitutes an assay and a run for the bioassay. Multiple dilutions (concentrations) of the Standard and one or more Test samples constitute a *replicate set* (also known as a minimal set), which contain a test substrate (e.g., group of animals or vessel of cells) at each dilution for each sample [Test(s) and Standard]. A *run* is defined as work performed during a period when the accuracy (trueness) and precision in the assay system can reasonably be expected to be stable. In practice, a run frequently consists of the work performed by a single analyst in one lab, with one set of equipment, in a short period of time (typically a day). An assay is the body of data used to assess similarity and estimate potency relative to a Standard for each Test sample in the assay. A run may contain multiple assays, a single assay, or part of an assay. Multiple assays may be combined to yield a reportable value for a sample. The reportable value is the value that is compared to a product specification.

In assays that involve groups at each dilution (e.g., 6 samples, each at 10 dilutions, in the non-edge wells of each of several 96-well cell culture plates) the groups (plates) constitute statistical *blocks* that should be elements in the assay and validation analyses (blocks are discussed in ⟨1032⟩). Within-block replicates for Test samples are rarely cost-effective. Blocks will not be further discussed in this chapter; more detailed discussion is found in ⟨1032⟩.

The amount of activity (potency) of the Standard is initially assigned a value of 1.0 or 100%, and the potency of the Test sample is calculated by comparing the concentration–response curves for the Test and Standard pair. This results in a unitless measure, which is the relative potency of the Test sample in reference to the potency of the Standard. In some cases the Standard is assigned a value according to another property such as protein concentration. In that case the potency of the Test sample is the relative potency times the assigned value of the Standard. An assumption of parallel-line or parallel-curve (e.g., four-parameter logistic) bioassays is that the dose–response curves that are generated using a Standard and a Test sample have similar (parallel) curve shape distinguished only by a horizontal shift in the log dose. For slope-ratio bioassays, curves generated for Standard and Test samples should be linear, pass through a common intercept, and differ only by their slopes. Information about how to assess parallelism is provided in general chapters ⟨1032⟩ and ⟨1034⟩.

In order to establish the *relative accuracy* and *range* of the bioassay, validation Test samples may be constructed using a dilution series of the Standard to assess dilutional linearity (linearity of the relationship between known and measured relative potency). In addition, the validation study should yield a representative estimate of the variability of the relative potency determination. Although robustness studies are usually performed during bioassay development, key factors in these studies such as incubation time and temperature and, for cell-based bioassays, cell passage number and cell number may be included in the validation, particularly if they interact with another factor that is introduced during the validation (e.g., a temperature sensitive reagent that varies in its sensitivity from lot-to-lot). Because of potential influences on the bioassay from inter-run factors such as multiple analysts, instruments, or reagent sources, the design of the bioassay validation should include consideration of these factors. The variability of potency from these combined elements defines the *intermediate precision* (IP) of the bioassay. An appropriate study of the variability of the potency values obtained, including the impact of intra-assay and inter-run factors, can help the laboratory confirm an adequate testing strategy and forecast the inherent variability of the *reportable value* (which may be the average of multiple potency determinations). Variability estimates can also be utilized to establish the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. (See section 3.4 *Use of Validation Results for Bioassay Characterization.*)

Demonstrating specificity (also known as selectivity) requires evidence of lack of influence from matrix components such as manufacturing process components or degradation products so that measurements quantify the target molecule only. Other analytical methods may complement a bioassay in measuring or identifying other components in a sample.

2.1 Bioassay Validation Protocol

A bioassay validation protocol should include the number and types of samples that will be studied in the validation; the study design, including inter-run and intra-run factors; the replication strategy; the intended validation parameters and justified target acceptance criteria for each parameter; and a proposed data-analysis plan. Note that in regard to satisfying acceptance criteria, failure to find a statistically significant effect is not an appropriate basis for defining acceptable performance in a bioassay; conformance to acceptance criteria may be better evaluated using an equivalence approach.

In addition, assay, run, and sample acceptance criteria such as system suitability and similarity should be specified before performing the validation. Depending on the extent of development of the bioassay, these may be proposed as tentative and

can be updated with data from the validation. Assay, run, or sample failures may be reassessed according to criteria which have been defined in the validation protocol and, with sound justification, included in the overall validation assessment. Additional validation trials may be required in order to support changes to the method.

The bioassay validation protocol should include target acceptance criteria for the proposed validation parameters. Steps to be taken upon failure to meet a target acceptance criterion should be specified in the validation protocol, and may result in a limit on the range of potencies that can be measured in the bioassay or a modification to the replication strategy in the bioassay procedure.

2.2 Documentation of Bioassay Validation Results

Bioassay validation results should be documented in a bioassay validation report. The validation report should support the conclusion that the method is fit for use or should indicate corrective action (such as an increase in the replication strategy) that will be undertaken to generate sufficiently reliable results to achieve fitness for use. The report could include the raw data and intermediate results (e.g., variance component estimates should be provided in addition to overall intermediate precision) which would facilitate reproduction of the bioassay validation analysis by an independent reviewer. Estimates of validation parameters should be reported at each level and overall as appropriate. Deviations from the validation protocol should be documented with justification. The conclusions from the study should be clearly described with references to follow-up action as necessary. Follow-up action can include amendment of system or sample suitability criteria or modification of the bioassay replication strategy. Reference to prevalidation experiments may be included as part of the validation study report. Prevalidation experiments may include robustness experiments, where bioassay parameters have been identified and ranges have been established for significant parameters, and also may include qualification experiments, where the final procedure has been performed to confirm satisfactory performance in routine operation. Conclusions from prevalidation and qualification experiments performed during development contribute to the description of the operating characteristics of the bioassay procedure.

2.3 Bioassay Validation Design

The biological assay validation should include samples that are representative of materials that will be tested in the bioassay and should effectively establish the performance characteristics of the procedure. For *relative accuracy,* sample relative potency levels that bracket the range of potencies that may be tested in the bioassay should be used. Thus samples that span a wide range of potencies might be studied for a drug or biological with a wide specification range or for a product that is inherently unstable, but a narrower range can be used for a more durable product. A minimum of three potency levels is required, and five are recommended for a reliable assessment. If the validation criteria for relative accuracy and IP are satisfied, the potency levels chosen will constitute the *range* of the bioassay. A limited range will result from levels that fail to meet their target acceptance criteria. Samples may also be generated for the bioassay validation by stressing a sample to a level that might be observed in routine practice (i.e., stability investigations). Additionally, the influences of the sample matrix (excipients, process constituents, or combination components) can be studied strategically by intentionally varying these together with the target *analyte*, using a multifactorial approach. Often this will have been done during development, prior to generating release and stability data.

The bioassay validation design should consider all facets of the measurement process. Sources of bioassay measurement variability include sample preparation, intra-run factors, and inter-run factors. Representative estimation of bioassay variability necessitates consideration of these factors. Test sample and Standard preparation should be performed independently during each validation run.

The replication strategy used in the validation should reflect knowledge of the factors that might influence the measurement of potency. Intra-run variability may be affected by bioassay operating factors that are usually set during development (temperature, pH, incubation times, etc.); by the bioassay design (number of animals, number of dilutions, replicates per dilution, dilution spacing, etc.); by the assay acceptance and sample acceptance criteria; and by the statistical analysis (where the primary endpoints are the similarity assessment for each sample and potency estimates for the reference samples). Operating restrictions and bioassay design (intra- and inter-run formulae that result in a *reportable value* for a test material) are usually specified during development and may become a part of the bioassay operating procedure. IP is studied by independent *runs* of the procedure, perhaps using an experimental design that alters those factors that may have an impact on the performance of the procedure. Experiments (including those that implement formalized design of experiments [DOE]) with nested or crossed design structure can reveal important sources of variability in the procedure, as well as ensure a representative estimate of long-term variability. During the validation it is not necessary to employ the format required to achieve the reportable value for a Test sample. A well-designed validation experiment that combines both intra-run and inter-run sources of variability provides estimates of independent components of the bioassay variability. These components can be used to verify or forecast the variability of the bioassay format.

A thorough analysis of the validation data should include graphical and statistical summaries of the validation parameters' results and their conformance to target acceptance criteria. The analysis should follow the specifics of the data-analysis plan outlined in the validation protocol. In most cases, log relative potency should be analyzed in order to satisfy the assumptions of the statistical methods (see section 2.7 *Statistical Considerations, Scale of Analysis,*). Those assumptions include *normality* of the distribution from which the data were sampled and *homogeneity of variability* across the range of results observed in the

validation. These assumptions can be explored using graphical techniques such as box plots and probability plots. The assumption of normality can be investigated using statistical tests of normality across a suitably sized collection of historical results. Alternative methods of analysis should be sought when the assumptions can be challenged. *Confidence intervals* should be calculated for the validation parameters, using methods described here and in general chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩.

2.4 Validation Strategies for Bioassay Performance Characteristics

Parameters that should be verified in a bioassay are *relative accuracy, specificity,* IP (which incorporates repeatability), and *range.* Other parameters discussed in general chapter ⟨1225⟩ and ICH Q2(R1) such as detection limit and quantitation limit have not been included because they are usually not relevant to a bioassay that reports relative potency. These may be relevant, however, to the validation of an ancillary assay such as one used to score responders or measure response in conjunction with an *in vivo* potency assay. Likewise linearity is not part of bioassay validation, except as it relates to relative accuracy (dilutional linearity). There follow strategies for addressing bioassay validation parameters.

Relative Accuracy

The *relative accuracy* of a relative potency bioassay is the relationship between measured relative potency and known relative potency. Relative accuracy in bioassay refers to a unit slope (slope = 1) between log measured relative potency and log known relative potency. The most common approach to demonstrating relative accuracy for relative potency bioassays is by construction of target potencies by dilution of the standard material or a Test sample with known potency. This type of study is often referred to as a *dilutional linearity study.* The results from a dilutional linearity study should be assessed using the estimated relative bias at individual levels and via a trend in *relative bias* across levels. The *relative bias* at individual levels is calculated as follows:

$$\text{Relative Bias} = 100 \cdot \left(\frac{\text{Measured Potency}}{\text{Target Potency}} - 1\right)\%$$

The trend in bias is measured by the estimated slope of log measured potency versus log target potency, which should be held to a target acceptance criterion. If there is no trend in *relative bias* across levels, the estimated *relative bias* at each level can be held to a prespecified target acceptance criterion that has been defined in the validation protocol (see section 3 *A Bioassay Validation Example*).

Specificity—For products or intermediates associated with complex matrices, specificity involves demonstrating lack of interference from matrix components or product-related components that can be expected to be present. This can be assessed via parallel dilution of the Standard with and without a spike addition of the potentially interfering compound. If the curves are similar and the potency conforms to expectations of a Standard-to-Standard comparison, the bioassay is specific against the compound. For these assessments both similarity and potency may be assessed using appropriate equivalence tests.

Specificity may also refer to the capacity of the bioassay to distinguish between different but related biopharmaceutical molecules. An understanding should be sought of the molecule and any related forms, and of opportunities for related molecules to be introduced into the bioassay.

Intermediate Precision—Because of potential influences on the bioassay by factors such as analysts, instruments, or reagent lots, the design of the bioassay validation should include evaluation of these factors. The overall variability from measurements taken under a variety of normal test conditions within one laboratory defines the IP of the bioassay. IP is the ICH and USP term for what is also commonly referred to as inter-run variability. IP measures the influence of factors that will vary over time after the bioassay is implemented. These influences are generally unavoidable and include factors like change in personnel (new analysts), receipt of new reagent lots, etc.

When the validation has been planned using multifactor DOE, the impact of each factor can first be explored graphically to establish important contributions to potency variability. The identification of important factors should lead to procedures that seek to control their effects, such as further restrictions on intra-assay operating conditions or strategic qualification procedures on inter-run factors such as analysts, instruments, and reagent lots.

Contributions of validation study factors to the overall IP of the bioassay can be determined by performing a *variance component analysis* on the validation results. *Variance component analysis* is best carried out using a statistical software package that is capable of performing a mixed-model analysis with restricted maximum likelihood estimation (REML).

A variance component analysis yields variance component estimates such as

$$\hat{\sigma}^2_{Intra}$$

and

$$\hat{\sigma}^2_{Inter}$$

corresponding to intra-run and inter-run variation. These can be used to estimate the IP of the bioassay, as well as the variability of the *reportable value* for different bioassay formats (format variability). IP expressed as percent *geometric* coefficient of variation (%GCV) is given by the following formula, in this case using the natural log of the relative potency in the analysis (see section 2.7 *Statistical Considerations, Scale of Analysis*):

$$\text{Intermediate Precision} = 100 \cdot \left(e^{\sqrt{\sigma^2_{\text{Inter}} + \sigma^2_{\text{Intra}}}} - 1\right)\%$$

The variability of the *reportable value* from testing performed with n replicate sets in each of k runs (*format variability*) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{\sigma^2_{\text{Inter}}/k + \sigma^2_{\text{Intra}}/(nk)}} - 1\right)\%$$

This formula can be used to determine a testing format suitable for various uses of the bioassay (e.g., release testing and stability evaluation).

Range—The *range* of the bioassay is defined as the true or known potencies for which it has been demonstrated that the analytical procedure has a suitable level of relative accuracy and IP. The range is normally derived from the dilutional linearity study and minimally should cover the product specification range for potency. For stability testing and to minimize having to dilute or concentrate hyper- or hypo-potent Test samples into the bioassay range, there is value in validating the bioassay over a broader range.

2.5 Validation Target Acceptance Criteria

The validation target acceptance criteria should be chosen to minimize the risks inherent in making decisions from bioassay measurements and to be reasonable in terms of the capability of the art. When there is an existing product specification, acceptance criteria can be justified on the basis of the risk that measurements may fall outside of the product specification. Considerations from a process capability (Cp) index can be used to inform bounds on the *relative bias* (RB) and the IP of the bioassay. This chapter uses the following Cpm index:

$$\text{Cpm} = \frac{\text{USL} - \text{LSL}}{6 \cdot \sqrt{\sigma^2_{\text{Product}} + \text{RB}^2 + \sigma^2_{\text{RA}}}}$$

where USL and LSL are the upper and lower release specification, RB is a bound on the degree of relative bias in the bioassay, and

$$\sigma^2_{\text{Product}}$$

and

$$\sigma^2_{\text{RA}}$$

are target product variance (i.e., lot-to-lot variability) and release assay variance (with associated format) respectively. (See section 3 *A Bioassay Validation Example* for an example of determination of

$$\sigma^2_{\text{RA}}$$

and Cpm.) This formulation requires prior knowledge regarding target product variability, or the inclusion of a random selection of lots to estimate this characteristic as part of the validation. Given limited understanding of assay performance, manufacturing history, and final specifications during development, this approach may be used simply as a guide for defining validation acceptance criteria.

The choice of a bound on Cpm is a business decision. The proportion of lots that are predicted to be outside their specification limits is a function of Cpm. Some laboratories require process capability corresponding to Cpm greater than or equal to 1.3. This corresponds to approximately a 1 in 10,000 chance that a lot with potency at the center of the specification range will be outside the specification limits.

When specifications have yet to be established for a product, a restriction on *relative bias* or IP can be formulated on the basis of the capability of the art of the bioassay methodology. For example, although chemical assays and immunoassays are often capable of achieving near single digit *percent coefficient of variation* (%CV, or percent relative standard deviation, %RSD), a more liberal restriction might be placed on bioassays, such as animal potency bioassays, that operate with much larger variability (measured as %GCV which can be compared to %CV; see *Appendix 1*). In this case the validation goal might be to *characterize* the method, using the validation results to establish an assay format that is predicted to yield reliable product measurements. A sound justification for target acceptance criteria or use of *characterization* should be included in the validation protocol.

2.6 Assay Maintenance

Once a bioassay has been validated it can be implemented. However, it is important to monitor its behavior over time. This is most easily accomplished by maintaining *statistical process control* (SPC) charts for suitable parameters of the Standard response curve and potency of assay QC samples. The purpose of these charts is to identify at an early stage any shift or drift in the bioassay. If a trend is observed in any SPC chart, the reason for the trend should be identified. If the resolution requires a modification to the bioassay or if a serious modification of the bioassay has occurred for other reasons (for example, a major technology change), the modified bioassay should be revalidated or linked to the original bioassay by an adequately designed bridging study with acceptance criteria that use equivalence testing.

2.7 Statistical Considerations

Several statistical considerations are associated with designing a bioassay validation and analyzing the data. These relate to the properties of bioassay measurements as well as the statistical tools that can be used to summarize and interpret bioassay validation results.

SCALE OF ANALYSIS

The scale of analysis of bioassay validation, where data are the relative potencies of samples in the validation study, must be considered in order to obtain reliable conclusions from the study. This chapter assumes that appropriate methods are already in place to reduce the raw bioassay response data to relative potency (as described in general chapter ⟨1034⟩). Relative potency measurements are typically nearly *log normally distributed. Log normally distributed* measurements are skewed and are characterized by *heterogeneity of variability*, where the standard deviation is proportional to the level of response. The statistical methods outlined in this chapter require that the data be symmetric, approximating a normal distribution, but some of the procedures require *homogeneity of variability* in measurements across the potency range. Typically, analysis of potency after log *transformation* generates data that more closely fulfill both of these requirements. The base of the log transformation does not matter as long as a consistent base is maintained throughout the analysis. Thus, for example, if the natural log (log to the base e) is used to transform relative potency measurements, summary results are converted back to the bioassay scale utilizing base e.

The distribution of potency measurements should be assessed as part of bioassay development (as described in ⟨1032⟩). If it is determined that potency measurements are normally distributed, the validation can be carried out using methods described in the general chapter *Validation of Compendial Procedures* ⟨1225⟩.

As a consequence of the usual (for parallel-line assays) log transformation of relative potency measurements, there are advantages if the levels selected for the validation study are evenly spaced on the log scale. An example with five levels would be 0.50, 0.71, 1.00, 1.41, and 2.00. Intermediate levels are obtained as the *geometric mean* of two adjacent levels. Thus for example, the mid-level between 0.50 and 1.0 is derived as follows:

$$\mathrm{GM} = \sqrt{0.50 \cdot 1.0} = 0.71$$

Likewise, summary measures of the validation are influenced by the log normal scale. Predicted response should be reported as the *geometric mean* of individual relative potency measurements, and variability expressed as %GCV. GCV is calculated as the anti-log of the standard deviation, S_{log}, of log transformed relative potency measurements. The formula is given by:

$$\mathrm{GCV} = \mathrm{antilog}(S_{log}) - 1$$

Variability is expressed as GCV rather than RSD of the log normal distribution in order to preserve continuity using the log transformation (see additional discussion in the *Appendix 1* to this chapter). Intervals that might be calculated from GCV will be consistent with intervals calculated from mean and standard deviation of log transformed data. *Table 1* presents an example of the calculation of geometric mean (GM) and associated RB, with %GCV for a series of relative potency measurements performed on samples tested at the 1.00 level. The log base e is used in the illustration.

Table 1. Illustration of calculations of GM and %GCV

RP[1]	ln RP	
1.1299	0.1221	
0.9261	−0.0768	
1.1299	0.1221	
1.0143	0.0142	
1.0027	0.0027	
1.0316	0.0311	
1.1321	0.1241	

[1] Relative potency (RP) is the geometric mean of duplicate potencies measured in the eight runs of the example given in *Table 4*.

Table 1. Illustration of calculations of GM and %GCV *(Continued)*

RP[1]	ln RP	
1.0499	0.0487	
Average	0.0485	GM = 1.0497
		RB = 4.97%
SD	0.0715	%GCV = 7.4%

[1] Relative potency (RP) is the geometric mean of duplicate potencies measured in the eight runs of the example given in *Table 4*.

Here the GM of the relative potency measurements is calculated as the anti-log of the average log relative potency measurements and then expressed as relative bias, the percent deviation from the target potency:

$$GM = e^{Average} = e^{0.0485} = 1.0497$$

$$RB = 100 \cdot \left(\frac{GM}{Target} - 1\right)\% = 100 \cdot \left(\frac{1.0497}{1.00} - 1\right)\% = 4.97\%$$

and the percent *geometric coefficient of variation* (%GCV) is calculated as:

$$\%GCV = 100 \cdot (e^{SD} - 1)\% = 100 \cdot (e^{0.0715} - 1)\% = 7.4\%$$

Note that the %GCV calculated for this illustration is not equal to the IP determined in the bioassay validation example for the 1.00 level (8.5%); see *Table 6*. This illustration utilizes the average of within-run replicates, while the IP in the validation example represents the variability of individual replicates.

Reporting Validation Results Using Confidence Intervals—Estimates of bioassay validation parameters should be presented as a *point estimate* together with a *confidence interval*. A *point estimate* is the numerical value obtained for the parameter, such as the GM or %GCV. A *confidence interval's* most common interpretation is as the likely range of the true value of the parameter. The previous example determines a 90% *confidence interval* for average log relative potency, CI_{ln}, as follows:

$$CI_{ln} = Average \pm t_{df} \cdot SD/\sqrt{n}$$
$$= 0.0485 \pm 1.89 \cdot 0.0715/\sqrt{8} = (0.0007, 0.0963)$$

For percent relative bias this is:

$$CI_{RB} = 100 \cdot \left(\frac{e^{0.0007}}{1.00} - 1\right)\%, 100 \cdot \left(\frac{e^{0.0963}}{1.00} - 1\right)\% = (0.07\%, 10.1\%)$$

The statistical constant (1.89) is from a t-table, with degrees of freedom (df) equal to the number of measurements minus one (df = 8 − 1 = 7). A confidence interval for IP or format variability can be formulated using methods for variance components; these methods are not covered in this general chapter.

Assessing Conformance to Acceptance Criteria—Bioassay validation results are compared to target acceptance criteria in order to demonstrate that the bioassay is fit for use. The process of establishing conformance of validation parameters to validation acceptance criteria should not be confused with establishing conformance of relative potency measurements to product specifications. Product specifications should inform the process of setting validation acceptance criteria.

A common practice is to apply acceptance criteria to the estimated validation parameter. This does not account, however, for the uncertainty in the estimated validation parameter. A solution is to hold the *confidence interval* on the validation parameter to the acceptance criterion. This is a standard statistical approach used to demonstrate conformance to expectation and is called an *equivalence test*. It should not be confused with the practice of performing a significance test, such as a t-test, which seeks to establish a difference from some target value (e.g., 0% relative bias). A significance test associated with a P-value > 0.05 (equivalent to a confidence interval that includes the target value for the parameter) indicates that there is insufficient evidence to conclude that the parameter is different from the target value. This is not the same as concluding that the parameter conforms to its target value. The study design may have too few *replicates*, or the validation data may be too variable to discover a meaningful difference from target. Additionally, a significance test may detect a small deviation from target that is of negligible importance. These scenarios are illustrated in *Figure 1*.

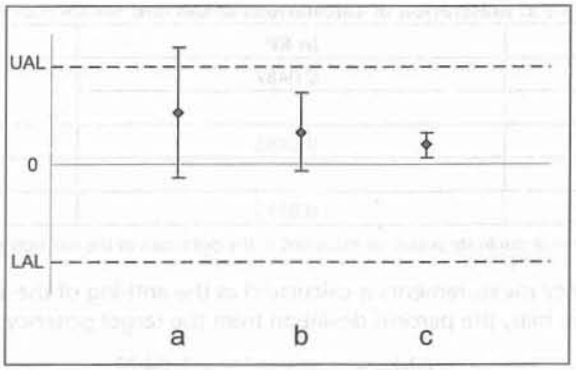


Figure 1. Use of confidence intervals to establish that validation results conform to an acceptance criterion.

The solid horizontal line represents the target value (perhaps 0% relative bias), and the dashed lines form the lower (LAL) and upper (UAL) acceptance limits. In scenario a, the confidence bound includes the target, and thus one could conclude there is insufficient evidence to conclude a difference from target (the significance test approach). However, although the *point estimate* (the solid diamond) falls within the acceptance range, the interval extends outside the range, which signifies that the true relative bias may be outside the acceptable range. In scenario b, the interval falls within the acceptance range, signifying conformance to the acceptance criterion. The interval in scenario c also falls within the acceptance range but excludes the target. Thus, for scenario c, although the difference of the point estimate from the target is statistically significant, c is acceptable because the confidence interval falls within the target acceptance limits.

Using the 90% confidence interval calculated previously, we can establish whether the bioassay has acceptable relative bias at the 1.00 level compared to a target acceptance criterion of no greater than +12%, for example. Because the 90% confidence interval for percent relative bias (0.07%, 10.1%) falls within the interval (100*[(1/1.12) − 1]%, 100*[(1.12/1) − 1]%) = (− 11%, 12%), we conclude that there is acceptable relative bias at the 1.00 level. Note that a 90% confidence interval is used in an equivalence test rather than a conventional 95% confidence interval. This is common practice and is the same as the *two one-sided tests* (TOST) approach used in pharmaceutical bioequivalence testing.

Risks in Decision-Making and Number of Validation Runs—The application of statistical tests, including the assessment of conformance of a validation parameter to its acceptance criteria, involves risks. One risk is that the parameter does not meet its acceptance criterion although the property associated with that parameter is satisfactory; another, the converse, is that the parameter meets its acceptance criterion although the parameter is truly unsatisfactory. A consideration related to these risks is sample size.

The two types of risk can be simultaneously controlled via strategic design, including choice of the number of *runs* that will be conducted in the validation. Specifically, the minimum number of *runs* needed to establish conformance to an acceptance criterion for relative bias is given by:

$$n \geq \frac{\left(t_{\alpha,df} + t_{\beta/2,df}\right)^2 \hat{\sigma}^2_{IP}}{\theta^2}$$

where $t_{\alpha,df}$ and $t_{\beta,df}$ are distributional points from a Student's t-distribution; α and β are the one-sided type I and type II errors, and represent the risks associated with drawing the wrong conclusion in the validation; df is the degrees of freedom associated with the study design (usually n − 1);

$$\hat{\sigma}^2_{IP}$$

is a preliminary estimate of IP; and θ is the acceptable deviation (target acceptance criterion).

For example, if the acceptance criterion for relative bias is ± 0.11 log (i.e., θ = 0.11), the bioassay variability is

$$\hat{\sigma}_{IP} = 0.076 \text{ log}$$

and $\alpha = \beta = 0.05$,

$$n \geq \frac{(1.89 + 2.36)^2\, 0.076^2}{0.11^2} \approx 8 \text{ runs}$$

Note that this formulation of sample size assumes no intrinsic bias in the bioassay. A more conservative solution includes some nonzero bias in the determination of a sample size. This results in a greater sample size to offset the impact of the bias on the conclusions of the validation. In the current example the sample size increases to 10 runs if one assumes an intrinsic bias equal to 2%. Note also that this calculation represents a recursive solution (because the degrees of freedom depend on n) requiring statistical software or an algorithm that employs iterative methodology.

Note further that the selection of α and β should be justified on the basis of the corresponding risks of drawing the wrong conclusion from the validation.

Modeling Validation Results Using Mixed Effects Models—Many analyses associated with bioassay validation must account for multiple design factors such as *fixed effects* (e.g., potency level), as well as *random effects* (e.g., analyst, run, and replicate). Statistical models composed of both *fixed* and *random effects* are called *mixed effects models* and usually require sophisticated statistical software for analysis. The results of the analysis may be summarized in an *analysis of variance* (ANOVA) table or a table of variance component estimates. The primary goal of the analysis is to estimate critical parameters rather than establish the significance of an effect. The modeling output provides parameter estimates together with their *standard errors* of estimates that can be utilized to establish conformance of a validation parameter to its acceptance criterion. Thus the average *relative bias* at each level is obtained as a portion of the analysis together with its associated variability. These compose a *confidence interval* that is compared to the acceptance criterion as described above. If variances across levels can be pooled, statistical modeling can also determine the overall *relative bias* and IP by combining information across levels performed in the validation. Similarly, mixed effects models can be used to obtain variance components for validation study factors and to combine results across validation study samples and levels.

Statistical Design—Statistical designs, such as multifactor DOE or *nesting*, can be used to organize assay and runs in a bioassay validation. It is useful to incorporate factors that are believed to influence the bioassay response and that vary during long-term use of the procedure into these designs. Using these methods of design, the sources of variability may be characterized and a strategic test plan to manage the variability of the bioassay may be developed.

Table 2 shows an example of a multifactor DOE that incorporates multiple analysts, multiple cell culture preparations, and multiple reagent lots into the validation plan.

Table 2. Example of a Multifactor DOE with 3 Factors

Run	Analyst	Cell Prep	Reagent Lot
1	1	1	1
2	1	1	2
3	1	2	1
4	1	2	2
5	2	1	1
6	2	1	2
7	2	2	1
8	2	2	2

In this design each analyst performs the bioassay with both cell preparations and both reagent lots. This is an example of a *full factorial* design because all combinations of the factors are performed in the validation study. To reduce the number of runs in the study, *fractional factorial* designs may be employed when more than three factors have been identified. For example, if it is practical for an analyst to perform four assays in a run, a split-unit design could be used with analysts as the whole-plot factor and cell preparation and reagent lot as sub-plot factors. Unlike screening experiments, the validation design should incorporate as many factors at as many levels as possible in order to obtain a representative estimate of IP. More than two levels of a factor should be employed in the design whenever possible. This may be accomplished in a less structured manner, without regard to strict factorial layout. Validation runs should be randomized whenever possible to mitigate the potential influences of run order or time.

Figure 2 illustrates an example of a validation using *nesting* (replicates nested within plate, plate nested within analyst).

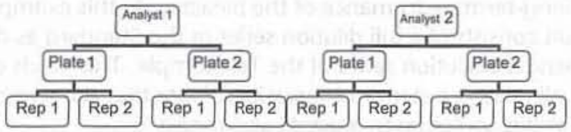


Figure 2. Example of a nested design using two analysts.

For both of these types of design as well as combinations of the two, components of variability can be estimated from the validation results. These components of variability can be used to identify significant sources of variability as well as to derive a bioassay *format* that meets the procedure's requirements for precision. It should be noted that significant sources of variability may have been identified during bioassay development. In this case the validation should confirm both the impact of these factors and the assay format that meets the requirement for precision.

Significant Figures—The number of significant figures in a reported result from a bioassay is related to the latter's precision. In general, a bioassay with %GCV between 2% and 20% will support two significant figures. The number of significant figures should not be confused with the number of decimal places—reported values equal to 1.2 and 0.12 have the same number (two) of significant figures. This standard of rounding is appropriate for log scaled measurements that have constant variation on the log scale and proportional rather than additive variability on the original scale (or the scale commonly used for interpretation). Note that rounding occurs at the end of a series of calculations when the final measurement is reported and used for

decision making such as conformance to specifications. Thus if the final measurement is a reportable value from multiple assays, rounding should not occur prior to determination of the reportable value. Likewise, specifications should be stated with the appropriate number of significant figures.

3. A BIOASSAY VALIDATION EXAMPLE

An example illustrates the principles described in this chapter. The bioassay will be used to support a specification range of 0.71 to 1.41 for the product. Using the Cpm described in section 2.5 *Validation Target Acceptance Criteria*, a table is derived showing the projected rate of OOS results for various restrictions on RB and IP. Cpm is calculated on the basis of the variability of a reportable value using three independent runs of the bioassay (see discussion of format variability, above). Product variability is assumed to be equal to 0 in the calculations. The laboratory may wish to include target product variability. An estimate of target product variability can be obtained from data from a product, for example, manufactured by a similar process.

Table 3. Cpm and Probability of OOS for Various Restrictions on RB and IP

LSL-USL	IP (%)	RB (%)	Cpm	Prob(OOS) (%)
0.71–1.41	20	20	0.54	10.5
0.71–1.41	8	12	0.94	0.48
0.71–1.41	10	5	1.55	0.0003

The calculation is illustrated for IP equal to 8% and relative bias equal to 12% (n = 3 runs):

$$\text{Cpm} = \frac{\ln(1.41) - \ln(0.71)}{6 \cdot \sqrt{[\ln(1.08)]^2/3 + [\ln(1.12)]^2}} = 0.94$$

$$\text{Prob(OOS)} = 2 \cdot \Phi(-3 \cdot 0.94) = 0.0048\ (0.48\%),$$

where Φ represents the standard normal cumulative distribution function.

From *Table 3*, acceptable performance (less than 1% chance of obtaining an OOS result due to bias and variability of the bioassay) can be expected if the IP is ≤8% and relative bias is ≤12%. The sample size formula given in section 2.7 *Statistical Considerations, Risks in Decision-Making and Number of Validation Runs* can be used to derive the number of runs required to establish conformance to an acceptance criterion for relative bias equal to 12% (using $\%GCV_{IP} = 8\%$; $\alpha = \beta = 0.05$):

$$n \geq \frac{(1.89 + 2.36)^2 \cdot [\ln(1.08)]^2}{[\ln(1.12)]^2} \approx 8 \text{ runs}$$

Thus eight runs would be needed in order to have a 95% chance of passing the target acceptance criterion for relative bias if the true relative bias is zero. Note that the calculation of sample size assumes that a singlet of the validation samples will be performed in each validation run. The use of multiple replication sets and/or multiple assays will provide valuable information that allows separate estimates for intra-run and inter-run variability, and will decrease the risk of failing to meet the validation target acceptance criteria.

Five levels of the target analyte are studied in the validation: 0.50, 0.71, 1.00, 1.41, and 2.00. Two runs at each level are generated by two trained analysts using two media lots. Other factors may be considered and incorporated into the design using a fractional factorial layout. The laboratory should strive to design the validation with as many levels of each factor as possible in order to best model the long-term performance of the bioassay. In this example each analyst performs two runs at each level using each media lot. A run consists of a full dilution series of the Standard as described in the bioassay's operating procedure, together with two independent dilution series of the Test sample. This yields duplicate measurements of relative potency in each run; see *Table 4* for all relative potency observations. Note that the two potency estimates at each level of potency in a run are not independent due to common analysts and media lots.

Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot

Media Lot/Analyst	1/1		1/2		2/1		2/2	
Run	1	2	1	2	1	2	1	2
0.50	0.5215	0.4532	0.5667	0.5054	0.5222	0.5179	0.5314	0.5112
0.50	0.5026	0.4497	0.5581	0.5350	0.5017	0.5077	0.5411	0.5488
0.71	0.7558	0.6689	0.6843	0.7050	0.6991	0.7463	0.6928	0.7400
0.71	0.7082	0.6182	0.8217	0.7143	0.6421	0.6877	0.7688	0.7399
1.00	1.1052	0.9774	1.1527	0.9901	1.0890	1.0314	1.1459	1.0273
1.00	1.1551	0.8774	1.1074	1.0391	0.9233	1.0318	1.1184	1.0730
1.41	1.5220	1.2811	1.5262	1.4476	1.4199	1.3471	1.4662	1.5035

General Chapters

Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot *(Continued)*

Media Lot/Analyst	1/1		1/2		2/1		2/2	
1.41	1.5164	1.3285	1.5584	1.4184	1.4025	1.4255	1.5495	1.5422
2.00	2.3529	1.8883	2.3501	2.2906	2.2402	2.1364	2.3711	2.0420
2.00	2.2307	1.9813	2.4013	2.1725	2.0966	2.1497	2.1708	2.3126

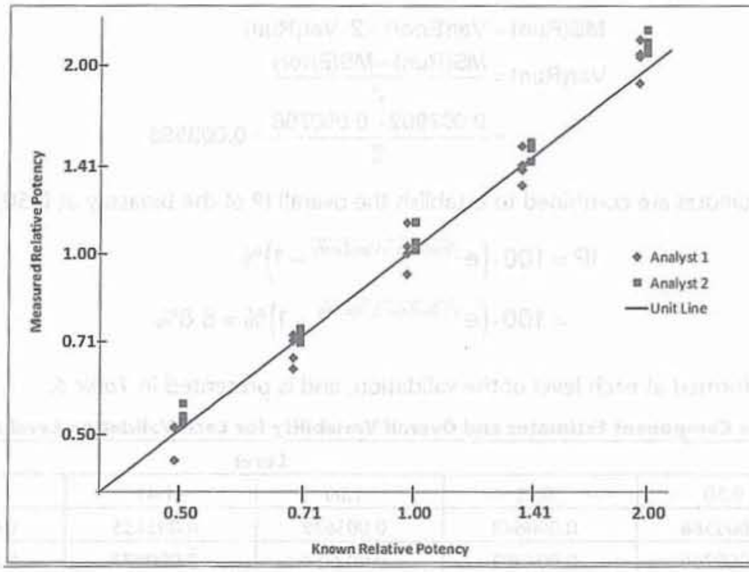


Figure 3. A plot of the validation results versus the sample levels.

A plot is used to reveal irregularities in the experimental results. In particular, a properly prepared plot can reveal a failure in agreement of validation results with validation levels, as well as *heterogeneity of variability* across levels (see discussion of the log transformation in section 2.7 *Statistical Considerations*). The example plot in *Figure 3* includes the unit line (line with slope equal to 1, passing through the origin). The analyst 1 and analyst 2 data are deliberately offset with respect to the expected potency to allow clear visualization and comparison of the data sets from each analyst.

A formal analysis of the validation data might be undertaken in the following steps: (1) an assessment of variability (IP) should precede an assessment of relative accuracy or specificity in order to establish conformance to the assumption that variances across sample levels can be pooled; and (2) *relative accuracy* is assessed either at separate levels or by a combined analysis, depending on how well the data across levels can be pooled. These steps are demonstrated using the example validation data, along with some details of the calculations for illustrative purposes. Note that the calculations illustrated in the following sections are appropriate only with a balanced dataset. Imbalanced designs or datasets with missing relative potency measurements should be analyzed using a mixed model analysis with restricted maximum likelihood estimation (REML).

3.1 Intermediate Precision

Data at each level can be analyzed using *variance component analysis*. With balanced data, as in this example, variance components can be determined from a standard one-way ANOVA. An example of the calculation performed at a single level (0.50) is presented in *Table 5*.

Table 5. Variance Component Analysis Performed on Log Relative Potency Measurements at the 0.5 Level

Source	df	Sum of Squares	Mean Square	Expected Mean Square
Run	7	0.055317	0.007902	Var(Error) + 2 Var(Run)
Error	8	0.006130	0.000766	Var(Error)
Corrected total	15	0.061447		
Variance Component Estimates				
Var(Run) = 0.003568				
Var(Error) = 0.000766				

The top of the table represents a standard ANOVA analysis. Analyst and media lot have not been included because of the small number of levels (2 levels) for each factor. The factor "Run" in this analysis represents the combined runs across the ana-

lyst by media lot combinations. The Expected Mean Square is the linear combination of variance components that generates the measured *mean square* for each source. The variance component estimates are derived by solving the equation "Expected Mean Square = Mean Square" for each component. To start, the *mean square* for Error estimates Var(Error), the within-run component of variability, is

$$\text{Var(Error)} = \text{MS(Error)} = 0.000766$$

The between-run component of variability, Var(Run), is subsequently calculated by setting the mean square for Run to the mathematical expression for the expected mean square, then solving the equation for Var(Run) as follows:

$$\text{MS(Run)} = \text{Var(Error)} + 2 \cdot \text{Var(Run)}$$

$$\text{Var(Run)} = \frac{\text{MS(Run)} - \text{MS(Error)}}{2}$$

$$= \frac{0.007902 - 0.000766}{2} = 0.003568$$

These *variance component estimates* are combined to establish the overall IP of the bioassay at 0.50:

$$\text{IP} = 100 \cdot \left(e^{\sqrt{\text{Var(Run)}+\text{Var(Error)}}} - 1\right)\%$$

$$= 100 \cdot \left(e^{\sqrt{0.003568+0.000766}} - 1\right)\% = 6.8\%$$

The same analysis was performed at each level of the validation, and is presented in *Table 6*.

Table 6. Variance Component Estimates and Overall Variability for Each Validation Level and the Average

	Level					
Component	0.50	0.71	1.00	1.41	2.00	Average
Var(Run)	0.003568	0.000648	0.003639	0.003135	0.002623	0.002723
Var(Error)	0.000766	0.004303	0.002954	0.000577	0.002258	0.002172
Overall	6.8%	7.3%	8.5%	6.3%	7.2%	7.2%

A combined analysis can be performed if the variance components are similar across levels. Typically a heuristic method is used for this assessment. One might hold the ratio of the maximum variance to the minimum variance to no greater than 10 (10 is used because of the limited number of runs performed in the validation). Here the ratios associated with the between-run variance component, 0.003639/0.000648 = 5.6, and the within-run component, 0.004303/0.000577 = 7.5, meet the 10-fold criterion. Had the ratio exceeded 10 and if this was due to excess variability in one or the other of the extremes in the levels tested, that extreme would be eliminated from further analysis and the range would be limited to exclude that level.

The analysis might proceed using statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis should account for any imbalance in the design, random effects such as analyst and media lot, and fixed effects such as level (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*). Variance components can be determined for analyst and media lot separately in order to characterize their contributions to the overall variability of the bioassay.

In the example, variance components can be averaged across levels to report the IP of the bioassay. This method of combining estimates is exact only if a balanced design has been employed in the validation (i.e., the same replication strategy at each level). A balanced design was employed for the example validation, so the IP can be reported as 7.2% GCV.

Because of the recommendation to report validation results with some measure of uncertainty, a one-sided 95% upper confidence bound can be calculated for the IP of the bioassay. The literature contains methods for calculating confidence bounds for variance components. The upper bound on IP for the bioassay example is 11.8% GCV. The upper confidence bound was not calculated at each level separately because of the limited data at an individual level relative to the overall study design.

3.2 Relative Accuracy

The analysis might proceed with an assessment of relative accuracy at each level. *Table 7* shows the average and 90% confidence interval of validation results in the log scale, as well as corresponding potency and relative bias.

Table 7. Average Potency and Relative Bias at Individual Levels

		Log Potency		Potency		Relative Bias	
Level	n[a]	Average	(90% CI)	Average	(90% CI)	Average	(90% CI)
0.50	8	−0.6613	(−0.7034, −0.6192)	0.52	(0.49, 0.54)	3.23%	(−1.02, 7.67)
0.71	8	−0.3419	(−0.3773, −0.3064)	0.71	(0.69, 0.74)	0.06%	(−3.42, 3.67)

[a] Analysis performed on averages of duplicates from each run.

[b] Calculation illustrated in section 2.7 *Statistical Considerations, Scale of Analysis.*

Table 7. Average Potency and Relative Bias at Individual Levels *(Continued)*

		Log Potency		Potency		Relative Bias	
Level	n[a]	Average	(90% CI)	Average	(90% CI)	Average	(90% CI)
1.00[b]	8	0.0485	(0.0006, 0.0964)	1.05	(1.00, 1.10)	4.97%	(0.06, 10.12)
1.41	8	0.3723	(0.3331, 0.4115)	1.45	(1.40, 1.51)	2.91%	(−1.04, 7.03)
2.00	8	0.7859	(0.7449, 0.8269)	2.19	(2.11, 2.29)	9.72%	(5.31, 14.32)

[a] Analysis performed on averages of duplicates from each run.
[b] Calculation illustrated in section 2.7 *Statistical Considerations, Scale of Analysis.*

The analysis has been performed on the average of the duplicates from each run (n = 8 runs) because duplicate measurements are correlated within a run by shared IP factors (analyst, media lot, and run in this case). A plot of relative bias versus level can be used to examine patterns in the experimental results and to establish conformance to the target acceptance criterion for relative bias (12%).

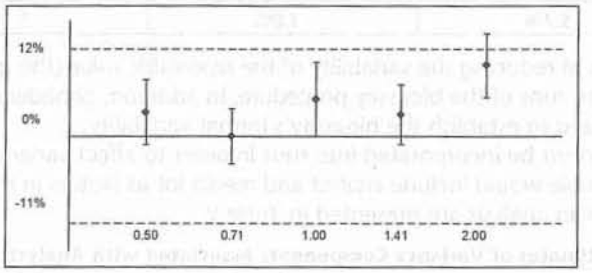


Figure 4. Plot of 90% confidence intervals for relative bias versus the acceptance criterion. Note lower acceptance criterion is equal to $100 \cdot [(1/1.12) - 1] = -11\%$.

Figure 4 shows an average positive bias across sample levels (i.e., the average relative bias is positive at all levels). This consistency is due in part to the lack of independence of bioassay results across levels. In addition there does not appear to be a trend in relative bias across levels. The latter would indicate that a comparison of samples with different measured relative potency (such as stability samples) is biased, resulting perhaps in an erroneous conclusion. Trend analysis can be performed using a regression of log relative potency versus log level. Introduction during the development of the bioassay validation protocol of an acceptance criterion on a trend in relative accuracy across the range can be considered.

After establishing that there is no meaningful trend across levels, the analysis proceeds with an assessment of the relative accuracy at each level. The bioassay has acceptable relative bias at levels from 0.50 to 1.41, yielding 90% confidence bounds (equivalent to a *two one-sided t-test*) that fall within the acceptance region of −11% to 12% relative bias. The 90% confidence interval at 2.0 falls outside the acceptance region, indicating that the relative bias may exceed 12%.

A combined analysis can be performed utilizing statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis accurately accounts for the validation study design. The analysis also accommodates *random effects* such as analyst, media lot, and run (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*).

3.3 Range

The conclusions derived from the assessment of IP and *relative accuracy* can be used to establish the bioassay's range that demonstrates satisfactory performance. Based on the acceptance criterion for IP equal to 8% GCV (see *Table 6*) and for relative bias equal to 12% (see *Table 7*), the range of the bioassay is 0.50 to 1.41. In this range, level 1.0 has a slightly higher than acceptable estimate of IP (8.5% versus the target acceptance criterion ≤8.0%), which may be due to the variability of the estimate that results from a small dataset. Because of this and other results in *Table 6*, one may conclude that satisfactory IP was demonstrated across the range.

3.4 Use of Validation Results for Bioassay Characterization

When the study has been performed to estimate the characteristics of the bioassay (characterization), the *variance component estimates* can also be used to predict the variability for different bioassay *formats* and thereby can determine a format that has a desired level of precision. The predicted variability for k independent *runs,* with n individual dilution series of the test preparation within a run, is given by the following formula for *format variability:*

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{Var(Run)/k + Var(Error)/(nk)}} - 1\right)$$

General Chapters

Using estimates of intra-run and inter-run variance components from *Table 6* [Var(Run) = 0.002723 and Var(Error) = 0.002172], if the bioassay is performed in three independent *runs*, the predicted variability of the *reportable value* (geometric mean of the relative potency results) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{0.002723/3 + 0.002172/(1 \cdot 3)}} - 1\right) = 4.1\%$$

This calculation can be expanded to include various combinations of *runs* and *minimal* sets (assuming that the numbers of samples, dilutions, and replicates in the minimal sets are held constant) within *runs* as shown in *Table 8.*

Table 8. Format Variability for Different Combinations of Number of Runs (k) and Number of Minimal Sets within Run (n)

	Number of Runs (k)			
Reps (n)	**1**	**2**	**3**	**6**
1	7.2%	5.1%	4.1%	2.9%
2	6.4%	4.5%	3.6%	2.6%
3	6.0%	4.2%	3.4%	2.4%
6	5.7%	4.0%	3.3%	2.3%

Clearly the most effective means of reducing the variability of the *reportable value* (the geometric mean potency across runs and minimal sets) is by independent runs of the bioassay procedure. In addition, confidence bounds on the variance components used to derive IP can be utilized to establish the bioassay's format variability.

Significant sources of variability must be incorporated into runs in order to effect variance reduction. A more thorough analysis of the bioassay validation example would include analyst and media lot as factors in the statistical model. Variance component estimates obtained from such an analysis are presented in *Table 9.*

Table 9. REML Estimates of Variance Components Associated with Analyst, Media Lot, and Run

Variance	Component Estimate
Var(Media Lot)	0.0000
Var(Analyst)	0.0014
Var(Analyst*Media Lot)	0.0000
Var(Run (Analyst*Media Lot))	0.0019
Var(Error)	0.0022

Identification of analyst as a significant bioassay factor should ideally be addressed during bioassay development. Nonetheless the laboratory may choose to address the apparent contribution of analyst-to-analyst variability through improved training or by using multiple analysts in formatting the assay for routine performance of the bioassay.

Estimates of intra-run and inter-run variability can also be used to determine the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. For k runs, with n minimal sets within each run, using an approximate two-sided critical value from the standard normal distribution with z = 2, the critical fold difference between reportable values for two samples that are tested in the same runs of the bioassay is given by:

$$\text{Critical Fold Difference} = e^{2 \cdot \sqrt{Var(Run)/k + Var(Error)/(nk)}}$$

When samples have been tested in different runs of the bioassay (such as long-term stability samples), the critical fold difference is given by (assuming the same format is used to test the two series of samples):

$$\text{Critical Fold Difference} = e^{2 \cdot \sqrt{2 \cdot [Var(Run)/k + Var(Error)/(nk)]}}$$

For comparison of samples the laboratory can choose a design (bioassay format) that has suitable precision to detect a practically meaningful fold difference between samples.

3.5 Confirmation of Intermediate Precision and Revalidation

The estimate of IP from the validation is highly uncertain because of the small number of runs performed. After the laboratory gains suitable experience with the bioassay, the estimate can be confirmed or updated by analysis of control sample measurements such as the variability of a positive control. This analysis can be done with the control prepared and tested like a Test sample (i.e., same or similar dilution series and replication strategy). This assessment should be made after sufficient assays have been performed to obtain an alternative estimate of the bioassay's intermediate precision, including implementation of changes (e.g., different analysts, different key reagent lots, and different cell preparations) associated with the standardized assay protocol. The reported IP of the bioassay should be modified as an amendment to the validation report if the assessment reveals a substantial disparity of results.

The bioassay should be revalidated whenever a substantial change is made to the method. This includes but is not limited to a change in technology or a change in readout. The revalidation may consist of a complete re-enactment of the bioassay validation or a bridging study that compares the current and the modified methods.

APPENDICES

Appendix 1: Measures of Location and Spread for Log Normally Distributed Variables

Two assumptions of common statistical procedures, such as ANOVA or confidence interval estimation, are (1) the variation in the bioassay response about its mean is normally distributed and (2) the standard deviation of the observed response values is constant over the range of responses that are of interest. Such responses are said to have a "normal distribution" and an "additive error structure". When these two conditions are not met, it may be useful to consider a transformation before using common statistical procedures.

The variation in bioassay responses is often found to be non-normal (skewed toward higher values) with a standard deviation approximately proportional (or nearly so) to the mean response. Such responses often have a "multiplicative error structure" and follow a "log normal distribution" with a percent coefficient of variation (%CV) that is constant across the response range of interest. In such cases, a log transformation of the bioassay response will be found to be approximately normal with a nearly constant standard deviation over the response range. After log transformation, then, the two assumptions are met, and common statistical procedures can be performed on the log transformed response. The following discussion presumes a log normal distribution for the bioassay response.

We refer to an observed bioassay response value, X, as being on the "original scale of measurement" and to the log transformed response, Y = log(X), as being on the "log transformed scale". Although common statistical procedures may be appropriate only on the log transformed scale, we can summarize bioassay response results by estimating measures of location (e.g., mean or median), measures of spread (e.g., standard deviation), or confidence intervals on either scale of measurement, as long as the scale being used is indicated. The %CV is useful on the original scale where it is constant over the response range. For the same reason, the standard deviation (SD) is relevant on the log transformed scale. There may be advantages to reporting statistical summaries on the basis of the log transformed (Y) scale. However, it is often informative to back transform the reported measures to the original scale of measurement (X).

For any given value of X, there is only one unique value of Y = log(X), and vice versa. Similarly for measures of location and spread, there is a unique one-to-one correspondence between measures of location and spread obtained on the original and log transformed scales. Further, just as there is a simple relationship between X and Y = log(X), there are relatively simple relationships that allow conversion between the corresponding measures on each scale, as indicated in *Table A-1* below. In the table, "Average" and "SD", wherever they appear, refer to measures calculated on the log transformed (Y) scale.

Table A-1. Comparison of Measures of Location and Spread

Measure		Scale of Measurement: Log Transformed (Y)	Scale of Measurement: Original (X)
Location		Mean (average)	Geometric mean (GM) $= \sqrt[n]{\prod_{i=1}^{n} X_i} = e^{\text{Average}}$
Spread		Standard deviation (SD)	Geometric standard deviation $(\text{GSD}) = e^{\text{SD}}$
Confidence intervals (k is an appropriate constant based on the t-distribution or large sample z approximation)	Lower	$\text{Average} - k \cdot \text{SD}/\sqrt{n}$	$\text{GM}/\text{GSD}^{k/\sqrt{n}}$
	Upper	$\text{Average} + k \cdot \text{SD}/\sqrt{n}$	$\text{GM} \cdot \text{GSD}^{k/\sqrt{n}}$
	Size	Width (upper − lower) $= 2 \cdot k \cdot \text{SD}/\sqrt{n}$	Ratio(upper/lower) $= \text{GSD}^{2k/\sqrt{n}}$
Percent coefficient of variation (%CV)		$\%\text{GCV} = 100 \cdot (\text{GSD} - 1)$	$\%\text{CV} = 100\sqrt{e^{\text{SD}^2} - 1} \lesssim \%\text{GCV}$

The geometric mean (GM) should not be misinterpreted as an estimate of the mean of the original scale (X) variable, but is instead an estimate of the median of X. The median is a more appropriate measure of location for variables with skewed error distributions such as the log normal, as well as symmetric error distributions where the median is equal to the mean.

Similarly, the geometric standard deviation (GSD) should not be misinterpreted as the standard deviation of the original scale (X) variable. GSD is, however, a useful multiplicative factor for obtaining confidence intervals on the original (X) scale that correspond to those on the log transformed (Y) scale, as shown in the above table. A GSD of 1 corresponds to no variation (SD of Y = 0). The ratio of the Upper to the Lower confidence bounds, on the untransformed (X) scale, will be equal to $\text{GSD}^{2k/\sqrt{n}}$, as can be seen from *Table A-1*.

The geometric coefficient of variation (%GCV) approximates the %CV on the original (X) scale when the %CV is below 20%. It is important not to confuse these different measures of spread. The %GCV is a measure relevant to the log transformed (Y) scale, and the %CV is a measure relevant to the original (X) scale. Depending on the preferred frame of reference, either or both measures may be useful.

Appendix 2: Information Sources

1. Limpert E, Stahel WA, Abbt M. (2001) Log-normal distributions across the sciences: keys and clues. BioScience 51(5): 341–252.
2. Kirkwood TBL. (1979) Geometric means and measures of dispersion. Biometrics 35: 908–909.
3. Bohidar NR. (1991) Determination of geometric standard deviation for dissolution. Drug Development and Industrial Pharmacy 17(10): 1381–1387.
4. Bohidar NR. (1993) Rebuttal to the "Reply". Drug Development and Industrial Pharmacy 19(3): 397–399.
5. Kirkwood TBL. (1993) Geometric standard deviation—reply to Bohidar. Drug Development and Industrial Pharmacy 19(3): 395–396.
6. ⟨1010⟩ Analytical data: interpretation and treatment. USP 34. In: USP34–NF 29. Vol. 1. Rockville (MD): United States Pharmacopeial Convention; c2011. p. 419.
7. Tan CY. (2005) RSD and other variability measures of the lognormal distribution. Pharmacopeial Forum 31(2): 653–655.

Appendix 3: Additional Sources of Information

Additional information and alternative methods can be found in the references listed below.

1. ASTM. Standard Practice for Using Significant Digits in Test Data to Determine Conformance with Specifications, ASTM E29-08. Conshohocken, PA: ASTM; 2008.
2. Berger R, Hsu J. Bioequivalence trials, intersection-union tests and equivalence confidence intervals. *Stat Sci* 1996;11(4): 283–319.
3. Burdick R, Graybill F. *Confidence Intervals on Variance Components*. New York: Marcel Dekker; 1992:28–39.
4. Haaland P. *Experimental Design in Biotechnology*. New York: Marcel Dekker; 1989;64–66.
5. Schofield TL. Assay validation. In: Chow SC, ed. *Encyclopedia of Biopharmaceutical Statistics*. 2nd ed. New York: Marcel Dekker; 2003.
6. Schofield TL. Assay development. In: Chow SC, ed. *Encyclopedia of Biopharmaceutical Statistics*. 2nd ed. New York: Marcel Dekker; 2003.
7. Winer B. *Statistical Principles in Experimental Design*. 2nd ed. New York: McGraw-Hill; 1971:244–251.

⟨1034⟩ ANALYSIS OF BIOLOGICAL ASSAYS

1. INTRODUCTION

Although advances in chemical characterization have reduced the reliance on bioassays for many products, bioassays are still essential for the determination of potency and the assurance of activity of many proteins, vaccines, complex mixtures, and products for cell and gene therapy, as well as for their role in monitoring the stability of biological products. The intended scope of general chapter *Analysis of Biological Assays* ⟨1034⟩ includes guidance for the analysis of results both of bioassays described in the *United States Pharmacopeia* (*USP*), and of non-USP bioassays that seek to conform to the qualities of bioassay analysis recommended by USP. Note the emphasis on analysis—design and validation are addressed in complementary chapters (*Development and Design of Bioassays* ⟨1032⟩ and *Biological Assay Validation* ⟨1033⟩, respectively).

Topics addressed in ⟨1034⟩ include statistical concepts and methods of analysis for the calculation of potency and confidence intervals for a variety of relative potency bioassays, including those referenced in *USP*. Chapter ⟨1034⟩ is intended for use primarily by those who do not have extensive training or experience in statistics and by statisticians who are not experienced in the analysis of bioassays. Sections that are primarily conceptual require only minimal statistics background. Most of the chapter and all the methods sections require that the nonstatistician be comfortable with statistics at least at the level of *USP* general chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and with linear regression. Most of sections 3.4 *Nonlinear Models for Quantitative Response* and 3.6 *Dichotomous (Quantal) Assays* require more extensive statistics background and thus are intended primarily for statisticians. In addition, ⟨1034⟩ introduces selected complex methods, the implementation of which requires the guidance of an experienced statistician.

Approaches in ⟨1034⟩ are recommended, recognizing the possibility that alternative procedures may be employed. Additionally, the information in ⟨1034⟩ is presented assuming that computers and suitable software will be used for data analysis. This view does not relieve the analyst of responsibility for the consequences of choices pertaining to bioassay design and analysis.

2. OVERVIEW OF ANALYSIS OF BIOASSAY DATA

Following is a set of steps that will help guide the analysis of a bioassay. This section presumes that decisions were made following a similar set of steps during development, checked during validation, and then not required routinely. Those steps and decisions are covered in general information chapter *Design and Development of Biological Assays* ⟨1032⟩. Section 3 *Analysis Models* provides details for the various models considered.

1. As a part of the chosen analysis, select the subset of data to be used in the determination of the relative potency using the prespecified scheme. Exclude only data known to result from technical problems such as contaminated wells, non-monotonic concentration–response curves, etc.
2. Fit the statistical model for detection of potential outliers, as chosen during development, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.
3. Determine which potential outliers are to be removed and fit the model to be used for suitability assessment. Usually, an investigation of outlier cause takes place before outlier removal. Some assay systems can make use of a statistical (noninvestigative) outlier removal rule, but removal on this basis should be rare. One approach to "rare" is to choose the outlier rule so that the expected number of false positive outlier identifications is no more than one; e.g., use a 1% test if the sample size is about 100. If a large number of outliers are found above that expected from the rule used, that calls into question the assay.
4. Assess system suitability. System suitability assesses whether the assay Standard preparation and any controls behaved in a manner consistent with past performance of the assay. If an assay (or a run) fails system suitability, the entire assay (or run) is discarded and no results are reported other than that the assay (or run) failed. Assessment of system suitability usually includes adequacy of the fit of the model used to assess similarity. For linear models, adequacy of the model may include assessment of the linearity of the Standard curve. If the suitability criterion for linearity of the Standard is not met, the exclusion of one or more extreme concentrations may result in the criterion being met. Examples of other possible system suitability criteria include background, positive controls, max/min, max/background, slope, IC_{50} (or EC_{50}), and variation around the fitted model.
5. Assess sample suitability for each Test sample. This is done to confirm that the data for each Test sample satisfy necessary assumptions. If a Test sample fails sample suitability, results for that sample are reported as "Fails Sample Suitability." Relative potencies for other Test samples in the assay may still be reported. Most prominent of sample suitability criteria is similarity, whether parallelism for parallel models or equivalence of intercepts for slope-ratio models. For nonlinear models, similarity assessment involves all curve parameters other than EC_{50} (or IC_{50}).
6. For those Test samples in the assay that meet the criterion for similarity to the Standard (i.e., sufficiently similar concentration–response curves or similar straight-line subsets of concentrations), calculate relative potency estimates assuming similarity between Test and Standard, i.e., by analyzing the Test and Standard data together using a model constrained to have exactly parallel lines or curves, or equal intercepts.
7. A single assay is often not sufficient to achieve a reportable value, and potency results from multiple assays can be combined into a single potency estimate. Repeat steps 1–6 multiple times, as specified in the assay protocol or monograph, before determining a final estimate of potency and a confidence interval.
8. Construct a variance estimate and a measure of uncertainty of the potency estimate (e.g., confidence interval). See section 4 *Confidence Intervals*.

A step not shown concerns replacement of missing data. Most modern statistical methodology and software do not require equal numbers at each combination of concentration and sample. Thus, unless otherwise directed by a specific monograph, analysts generally do not need to replace missing values.

3. ANALYSIS MODELS

A number of mathematical functions can be successfully used to describe a concentration–response relationship. The first consideration in choosing a model is the form of the assay response. Is it a number, a count, or a category such as Dead/Alive? The form will identify the possible models that can be considered.

Other considerations in choosing a model include the need to incorporate design elements in the model and the possible benefits of means models compared to regression models. For purposes of presenting the essentials of the model choices, section 3 *Analysis Models* assumes a completely randomized design so that there are no design elements to consider and presents the models in their regression form.

3.1 Quantitative and Qualitative Assay Responses

The terms *quantitative* and *qualitative* refer to the nature of the response of the assay used in constructing the concentration–response model. Assays with either quantitative or qualitative responses can be used to quantify product potency. Note that the *responses* of the assay at the concentrations measured are not the relative potency of the bioassay. Analysts should understand the differences among responses, concentration–response functions, and relative potency.

General Chapters

A quantitative response results in a number on a continuous scale. Common examples include spectrophotometric and luminescence responses, body weights and measurements, and data calculated relative to a standard curve (e.g., cytokine concentration). Models for quantitative responses can be linear or nonlinear (see sections 3.2–3.5).

A qualitative measurement results in a categorical response. For bioassay, qualitative responses are most often quantal, meaning they entail two possible categories such as Positive/Negative, 0/1, or Dead/Alive. Quantal responses may be reported as proportions (e.g., the proportion of animals in a group displaying a property). Quantal models are presented in section 3.6. Qualitative responses can have more than two possible categories, such as end-point titer assays. Models for more than two categories are not considered in this general chapter.

Assay responses can also be counts, such as number of plaques or colonies. Count responses are sometimes treated as quantitative, sometimes as qualitative, and sometimes models specific to integers are used. The choice is often based on the range of counts. If the count is mostly 0 and rarely greater than 1, the assay may be analyzed as quantal and the response is Any/None. If the counts are large and cover a wide range, such as 500 to 2500, then the assay may be analyzed as quantitative, possibly after transformation of the counts. A square root transformation of the count is often helpful in such analyses to better satisfy homogeneity of variances. If the range of counts includes or is near 0 but 0 is not the preponderant value, it may be preferable to use a model specific for integer responses. Poisson regression and negative binomial regression models are often good options. Models specific to integers will not be discussed further in this general chapter.

Assays with quantitative responses may be converted to quantal responses. For example, what may matter is whether some defined threshold is exceeded. The model could then be quantal—threshold exceeded or not. In general, assay systems have more precise estimates of potency if the model uses all the information in the response. Using above or below a threshold, rather than the measured quantitative responses, is likely to degrade the performance of an assay.

3.2 Overview of Models for Quantitative Responses

In quantitative assays, the measurement is a number on a continuous scale. Optical density values from plate-based assays are such measurements. Models for quantitative assays can be linear or nonlinear. Although the two display an apparent difference in levels of complexity, parallel-line (linear) and parallel-curve (nonlinear) models share many commonalities. Because of the different form of the equations, slope-ratio assays are considered separately (section 3.5 *Slope-Ratio Concentration–Response Models*).

Assumptions—The basic parallel-line, parallel-curve, and slope-ratio models share some assumptions. All include a residual term, e, that represents error (variability) which is assumed to be independent from measurement to measurement and to have constant variance from concentration to concentration and sample to sample. Often the residual term is assumed to have a normal distribution as well. The assumptions of independence and equal variances are commonly violated, so the goal in analysis is to incorporate the lack of independence and the unequal variances into the statistical model or the method of estimation.

Lack of independence often arises because of the design or conduct of the assay. For example, if the assay consists of responses from multiple plates, observations from the same plate are likely to share some common influence that is not shared with observations from other plates. This is an example of intraplate correlation. A simple approach for dealing with this lack of independence is to include a block term in the statistical model for plate. With three or more plates this should be a random effects term so that we obtain an estimate of plate-to-plate variability.

In general, the model needs to closely reflect the design. The basic model equations given in sections 3.3–3.5 apply only to completely randomized designs. Any other design will mean additional terms in the statistical model. For example, if plates or portions of plates are used as blocks, one will need terms for blocks.

Calculation of Potency—A primary assumption underlying methods used for the calculation of relative potency is that of similarity. Two preparations are similar if they contain the same effective constituent or same effective constituents in the same proportions. If this condition holds, the Test preparation behaves as a dilution (or concentration) of the Standard preparation. Similarity can be represented mathematically as follows. Let F_T be the concentration–response function for the Test, and let F_S be the concentration–response function for the Standard. The underlying mathematical model for similarity is:

$$F_T(z) = F_S(\rho z), \qquad [3.1]$$

where z represents the concentration and ρ represents the relative potency of the Test sample relative to the Standard sample.

Methods for estimating ρ in some common concentration–response models are discussed below. For linear models, the distinction between parallel-line models (section 3.3 *Parallel-Line Models for Quantitative Response*) and slope-ratio models (section 3.5 *Slope-Ratio Concentration–Response Models*) is based on whether a straight-line fit to log concentration or concentration yields better agreement between the model and the data over the range of concentrations of interest.

3.3 Parallel-Line Models for Quantitative Responses

In this section, a linear model refers to a concentration–response relationship, which is a straight-line (linear) function between the logarithm of concentration, x, and the response, y. y may be the response in the scale as measured or a transformation of the response. The functional form of this relationship is y = a + bx. Straight-line fits may be used for portions of nonlin-

ear concentration–response curves, although doing so requires a method for selecting the concentrations to use for each of the Standard and Test samples (see ⟨1032⟩).

Means Model versus Regression—A linear concentration–response model is most often analyzed with least squares regression. Such an analysis results in estimates of the unknown coefficients (intercepts and slope) and their standard errors, as well as measures of the goodness of fit [e.g., R^2 and root-mean-square error (RMSE)].

Linear regression works best where all concentrations can be used and there is negligible curvature in the concentration–response data. Another statistical method for analyzing linear concentration–response curves is the *means model*. This is an analysis of variance (ANOVA) method that offers some advantages, particularly when one or more concentrations from one or more samples are not used to estimate potency. Because a means model includes a separate mean for each unique combination of sample and dose (as well as block or other effects associated with the design structure) it is equivalent to a saturated polynomial regression model. Hence, a means model provides an estimate of error that is independent of regression lack of fit. In contrast, a regression residual based estimate of error is a mixture of the assay error, as estimated by the means model, combined with lack of fit of the regression model. At least in this sense, the means model error is a better estimate of the residual error variation in an assay system.

Parallel-Line Concentration–Response Models—If the general concentration–response model (3.1 *Quantitative and Qualitative Assay Responses*) can be made linear in x = log(z), the resulting equation is then:

$$y = \alpha + \beta \log(z) + e = \alpha + \beta x + e,$$

where e is the residual or error term, and the intercept, α, and slope, β, will differ between Test and Standard. With the parallelism (equal slopes) assumption, the model becomes

$$\begin{aligned} y_S &= \alpha + \beta \log(z) + e = \alpha_S + \beta x + e \\ y_T &= \alpha + \beta \log(\rho z) + e = [\alpha + \beta \log(\rho)] + \beta x + e = \alpha_T + \beta x + e, \end{aligned} \qquad [3.2]$$

where S denotes Standard, T denotes Test, $\alpha_S = \alpha$ is the y-intercept for the Standard, and $\alpha_T = \alpha + \beta \log(\rho)$ is the y-intercept for the Test (see *Figure 3.1*).

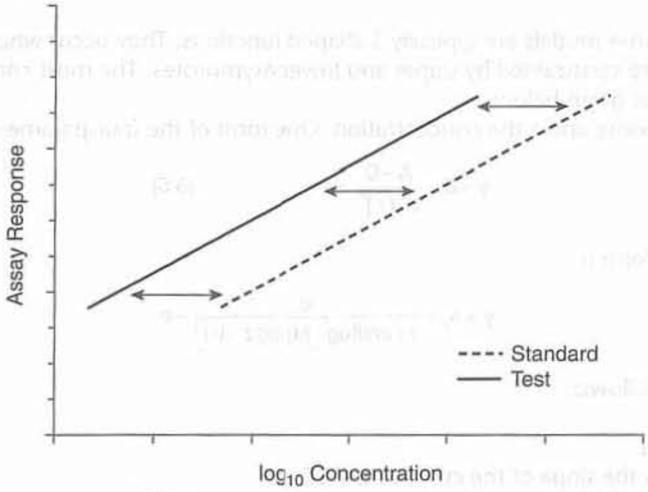


Figure 3.1. Example of parallel-line model.

Where concentration–response lines are parallel, as shown in *Figure 3.1*, a separation or horizontal shift indicates a difference in the level of biological activity being assayed. This horizontal difference is numerically log(ρ), the logarithm of the relative potency, and is found as the vertical distance between the lines α_T and α_S divided by the slope, β. The relative potency is then

$$\rho = \text{antilog}\left(\frac{\alpha_T - \alpha_S}{\beta}\right)$$

Estimation of Parallel-line Models—Parallel-line models are fit by the method of least squares. If the equal variance assumption holds, the parameters of equation [3.2] are chosen to minimize

$$\sum \left(y - \hat{\alpha}_S - \hat{\delta}T - \hat{\beta}x\right)^2 \qquad [3.3]$$

where the carets denote estimates. This is a linear regression with two independent variables, T and x, where T is a variable that equals 1 for observations from the Test and 0 for observations from the Standard. The summation in equation [3.3] is over all observations of the Test and Standard. If the equal variance assumption does not hold but the variance is known to be inversely proportional to a value, w, that does not depend on the current responses, the y's, and can be determined for each observation, then the method is weighted least squares

$$\sum w\left(y-\hat{\alpha}_S-\hat{\delta}T-\hat{\beta}x\right)^2 \qquad [3.4]$$

Equation 3.4 is appropriate only if the weights are determined without using the response, the y's, from the current data (see ⟨1032⟩ for guidance in determining weights). In equations [3.3] and [3.4] β is the same as the β in equation [3.2] and $\delta = \alpha_T - \alpha_S = \beta \log \rho$. So, the estimate of the relative potency, ρ, is

$$\hat{\rho} = \text{antilog}\left(\hat{\delta}/\hat{\beta}\right)$$

Commonly available statistical software and spreadsheets provide routines for least squares. Not all software can provide weighted analyses.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to δ/β.

Measurement of Nonparallelism—Parallelism for linear models is assessed by considering the difference or ratio of the two slopes. For the difference, this can be done by fitting the regression model,

$$y = \alpha_S + \delta T + \beta_S x + \gamma x T + e$$

where $\delta = \alpha_T - \alpha_S$, $\gamma = \beta_T - \beta_S$, and T = 1 for Test data and T = 0 for Standard data. Then use the standard t-distribution confidence interval for γ. For the ratio of slopes, fit

$$y = \alpha_S + \delta T + \beta_S x(1 - T) + \beta_T x T + e$$

and use Fieller's Theorem, equation [4.3], to obtain a confidence interval for β_T/β_S.

3.4 Nonlinear Models for Quantitative Responses

Nonlinear concentration–response models are typically S-shaped functions. They occur when the range of concentrations is wide enough so that responses are constrained by upper and lower asymptotes. The most common of these models is the four-parameter logistic function as given below.

Let y denote the observed response and z the concentration. One form of the four-parameter logistic model is

$$y = D + \frac{A-D}{1+\left(\frac{z}{C}\right)^B} + e \qquad [3.5]$$

One alternative, but equivalent, form is

$$y = a_0 + \frac{d}{1+\text{antilog}\left[M(\log z - b)\right]} + e$$

The two forms correspond as follows:

- Lower asymptote: $D = a_0$
- Upper asymptote: $A = a_0 + d$
- Steepness: B = M (related to the slope of the curve at the EC_{50})
- Effective concentration 50% (EC_{50}): C = antilog(b) (may also be termed ED_{50}).

Any convenient base for logarithms is suitable; it is often convenient to work in log base 2, particularly when concentrations are twofold apart.

The four-parameter logistic curve is symmetric around the EC_{50} when plotted against log concentration because the rates of approach to the upper and lower asymptotes are the same (see *Figure 3.2*). For assays where this symmetry does not hold, asymmetrical model functions may be applied. These models are not considered further in this general chapter.

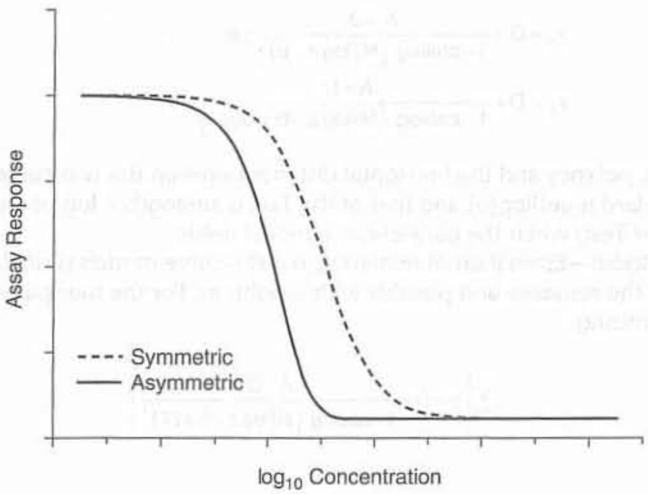


Figure 3.2. Examples of symmetric (four-parameter logistic) and asymmetric sigmoids.

In many assays the analyst has a number of strategic choices to make during assay development (see *Development and Design of Biological Assays* ⟨1032⟩). For example, the responses could be modeled using a transformed response to a four-parameter logistic curve, or the responses could be weighted and fit to an asymmetric sigmoid curve. Also, it is often important to include terms in the model (often random effects) to address variation in the responses (or parameters of the response) associated with blocks or experimental units in the design of the assay. For simple assays where observations are independent, these strategic choices are fairly straightforward. For assays performed with grouped dilutions (as with multichannel pipets), assays with serial dilutions, or assay designs that include blocks (as with multiple plates per assay), it is usually a serious violation of the statistical assumptions to ignore the design structure. For such assays, a good approach involves a transformation that approximates a solution to non-constant variance, non-normality, and asymmetry combined with a model that captures the important parts of the design structure.

Parallel-Curve Concentration–Response Models—The concept of parallelism is not restricted to linear models. For nonlinear curves, parallel or similar means the concentration–response curves can be superimposed following a horizontal displacement of one of the curves, as shown in *Figure 3.3* for four-parameter logistic curves. In terms of the parameters of equation [3.5], this means the values of A, D, and B for the Test are the same as for the Standard.

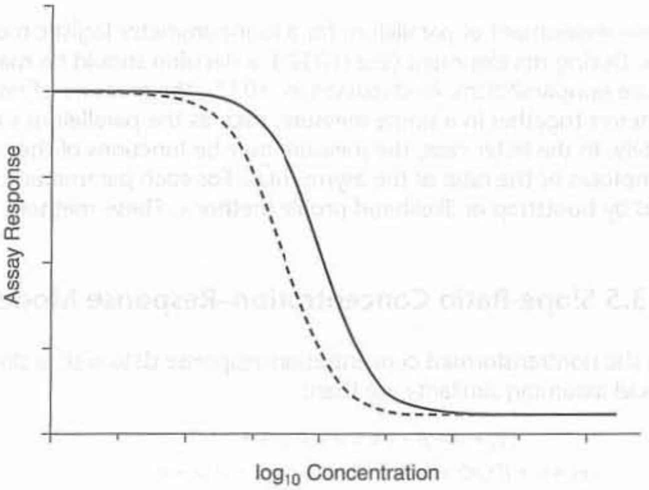


Figure 3.3. Example of parallel curves from a nonlinear model.

The equations corresponding to the figure (with error term, e, added) are

$$y_S = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e$$

$$y_T = D + \frac{A - D}{1 + \left(\frac{\rho z}{C}\right)^B} + e$$

or

$$y_S = D + \frac{A - D}{1 + \text{antilog}\left[M(\log z - b)\right]} + e$$

$$y_T = D + \frac{A - D}{1 + \text{antilog}\left[M(\log z - b + \log\rho)\right]} + e$$

Log ρ is the log of the relative potency and the horizontal distance between the two curves, just as for the parallel-line model. Because the EC_{50} of the standard is antilog(b) and that of the Test is antilog(b – log ρ) = antilog(b)/ρ, the relative potency is the ratio of EC_{50}'s (standard over Test) when the parallel-curve model holds.

Estimation of Parallel-Curve Models—Estimation of nonlinear, parallel-curve models is similar to that for parallel-line models, possibly after transformation of the response and possibly with weighting. For the four-parameter logistic model, the parameter estimates are found by minimizing:

$$\sum\left(y - \hat{D} - \frac{\hat{A} - \hat{D}}{1 + \text{antilog}\left[\hat{M}\left(\log z - \hat{b} + \hat{r}T\right)\right]}\right)^2$$

without weighting, or

$$\sum w\left(y - \hat{D} - \frac{\hat{A} - \hat{D}}{1 + \text{antilog}\left[\hat{M}\left(\log z - \hat{b} + \hat{r}T\right)\right]}\right)^2 \quad [3.6]$$

with weighting. (As for equation [3.4], equation [3.6] is appropriate only if the weights are determined without using the responses, y's, from the current data.) In either case, the estimate of r is the estimate of the log of the relative potency. For some software, it may be easier to work with d = A – D.

The parameters of the four-parameter logistic function and those of the asymmetric sigmoid models cannot be found with ordinary (linear) least squares regression routines. Computer programs with nonlinear estimation techniques must be used.

Analysts should not use the nonlinear regression fit to assess parallelism or estimate potency if any of the following are present: a) inadequate asymptote information is available; or b) a comparison of pooled error(s) from nonlinear regression to pooled error(s) from a means model shows that the nonlinear model does not fit well; or c) other appropriate measures of goodness of fit show that the nonlinear model is not appropriate (e.g., residual plots show evidence of a "hook").

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, advanced techniques, such as likelihood profiles or bootstrapping are needed to obtain a confidence interval for the log relative potency, r.

Measurement of Nonparallelism—Assessment of parallelism for a four-parameter logistic model means assessing the slope parameter and the two asymptotes. During development (see ⟨1032⟩), a decision should be made regarding which parameters are important and how to measure nonparallelism. As discussed in ⟨1032⟩, the measure of nonsimilarity may be a composite measure that considers all parameters together in a single measure, such as the parallelism sum of squares (see ⟨1032⟩), or may consider each parameter separately. In the latter case, the measure may be functions of the parameters, such as an asymptote divided by the difference of asymptotes or the ratio of the asymptotes. For each parameter (or function of parameters), confidence intervals can be computed by bootstrap or likelihood profile methods. These methods are not presented in this general chapter.

3.5 Slope-Ratio Concentration–Response Models

If a straight-line regression fits the nontransformed concentration–response data well, a slope-ratio model may be used. The equations for the slope-ratio model assuming similarity are then:

$$y_S = \alpha + \beta z + e = \alpha + \beta_S z + e$$
$$y_T = \alpha + \beta(\rho z) + e = \alpha + \beta_S \rho z + e = \alpha + \beta_T z + e \quad [3.7]$$

An identifying characteristic of a slope-ratio concentration–response model that can be seen in the results of a ranging study is that the lines for different potencies from a ranging study have the same intercept and different slopes. Thus, a graph of the ranging study resembles a fan. *Figure 3.4* shows an example of a slope-ratio concentration–response model. Note that the common intercept need not be at the origin.

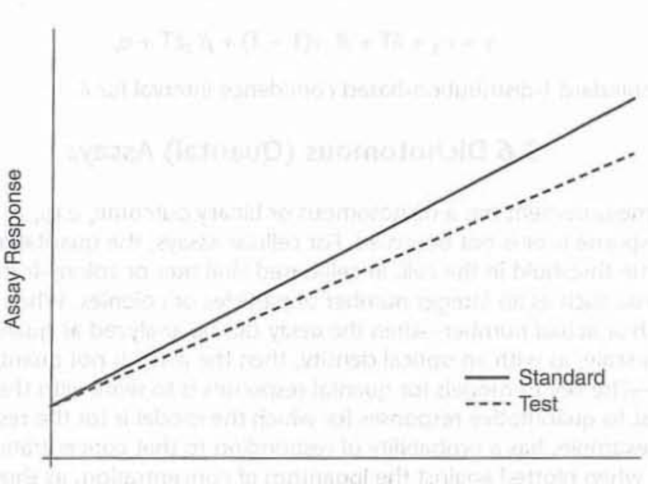


Figure 3.4. Example of slope-ratio model.

An assay with a slope-ratio concentration–response model for measuring relative potency consists, at a minimum, of one Standard sample and one Test sample, each measured at one or more concentrations and, usually, a measured response with no sample (zero concentration). Because the concentrations are not log transformed, they are typically equally spaced on the original, rather than log, scale. The model consists of one common intercept, a slope for the Test sample results, and a slope for the Standard sample results as in equation [3.7]. The relative potency is then found from the ratio of the slopes:

$$\text{Relative Potency} = \text{Test sample slope/Standard sample slope} = \beta\rho/\beta = \rho$$

Assumptions for and Estimation of Slope-Ratio Models—The assumptions for the slope-ratio model are the same as for parallel-line models: The residual terms are independent, have constant variance, and may need to have a normal distribution. The method of estimation is also least squares. This may be implemented either with or without weighting, as demonstrated in equations [3.8] and [3.9], respectively.

$$\sum\left(y-\hat{\alpha}-\hat{\beta}_S z(1-T)-\hat{\beta}_T zT\right)^2 \qquad [3.8]$$

$$\sum w\left(y-\hat{\alpha}-\hat{\beta}_S z(1-T)-\hat{\beta}_T zT\right)^2 \qquad [3.9]$$

Equation [3.9] is appropriate only if the weights are determined without using the response, the y's, from the current data. This is a linear regression with two independent variables, z(1 − T) and zT, where T = 1 for Test data and T = 0 for Standard data. β_T is the estimated slope for the Test, β_S the estimated slope for the Standard, and then the estimate of relative potency is

$$R = \hat{\beta}_T / \hat{\beta}_S$$

Because the slope-ratio model is a linear regression model, most statistical packages and spreadsheets can be used to obtain the relative potency estimate. In some assay systems, it is sometimes appropriate to omit the zero concentration (e.g., if the no-dose controls are handled differently in the assay) and at times one or more of the high concentrations (e.g., if there is a hook effect where the highest concentrations do not have the highest responses). The discussion about using a means model and selecting subsets of concentrations for straight parallel-line bioassays applies to slope-ratio assays as well.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to

$$\hat{\beta}_T / \hat{\beta}_S$$

Measurement of Nonsimilarity—For slope-ratio models, statistical similarity corresponds to equal intercepts for the Standard and Test. To assess the similarity assumption it is necessary to have at least two nonzero concentrations for each sample. If the intercepts are not equal, equation [3.7] becomes

$$y_S = \alpha_S + \beta_S z + e$$
$$y_T = \alpha_T + \beta_T z + e$$

Departure from similarity is typically measured by the difference of intercepts, $\alpha_T - \alpha_S$. An easy way to obtain a confidence interval is to fit the model,

$$y = \alpha_S + \delta T + \beta_S z(1 - T) + \beta_T zT + e,$$

where $\delta = \alpha T - \alpha_S$ and use the standard t-distribution-based confidence interval for δ.

3.6 Dichotomous (Quantal) Assays

For quantal assays the assay measurement has a dichotomous or binary outcome, e.g., in animal assays the animal is dead or alive or a certain physiologic response is or is not observed. For cellular assays, the quantal response may be whether there is or is not a response beyond some threshold in the cell. In cell-based viral titer or colony-forming assays, the quantal response may be a limit of integer response such as an integer number of particles or colonies. When one can readily determine if any particles are present—but not their actual number—then the assay can be analyzed as quantal. Note that if the reaction can be quantitated on a continuous scale, as with an optical density, then the assay is not quantal.

Models for Quantal Analyses—The key to models for quantal responses is to work with the probability of a response (e.g., probability of death), in contrast to quantitative responses for which the model is for the response itself. For each concentration, z, a treated animal, as an example, has a probability of responding to that concentration, P(z). Often the curve P(z) can be approximated by a sigmoid when plotted against the logarithm of concentration, as shown in *Figure 3.5*. This curve shows that the probability of responding increases with concentration. The concentration that corresponds to a probability of 0.5 is the EC_{50}.

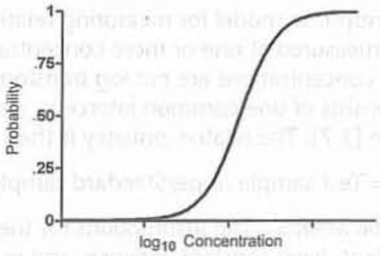


Figure 3.5. Example of sigmoid for P(z).

The sigmoid curve is usually modeled based on the normal or logistic distribution. If the normal distribution is used, the resulting analysis is termed probit analysis, and if the logistic is used the analysis is termed logit or logistic analysis. The probit and logit models are practically indistinguishable, and either is an acceptable choice. The choice may be based on the availability of software that meets the laboratory's analysis and reporting needs. Because software is more commonly available for logistic models (often under the term logistic regression) this discussion will focus on the use and interpretation of logit analysis. The considerations discussed in this section for logit analysis (using a logit transformation) apply as well to probit analysis (using a probit transformation).

Logit Model—The logit model for the probability of response, P(z), can be expressed in two equivalent forms. For the sigmoid,

$$P(z) = \frac{1}{1 + \text{antilog}\,[-\beta_0 - \beta_1 \log(z)]} = \frac{1}{1 + (z/ED_{50})^{-\beta_1}}$$

where $\log(ED_{50}) = -\beta_0/\beta_1$. An alternative form shows the relationship to linear models:

$$\text{logit transform of P} = \log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) \qquad [3.10]$$

The linear form is usually shown using natural logs and is a useful reminder that many of the considerations, in particular linearity and parallelism, discussed for parallel-line models in section 3.3 *Parallel-Line Models for Quantitative Responses* apply to quantal models as well.

For a logit analysis with Standard and Test preparations, let T be a variable that takes the value 1 for animals receiving the Test preparation and 0 for animals receiving the Standard. Assuming parallelism of the Test and Standard curves, the logit model for estimating relative potency is then:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T$$

The log of the relative potency of the Test compared to the Standard preparation is then β_2/β_1. The two curves in *Figure 3.6* show parallel Standard and Test sigmoids. (If the corresponding linear forms equation [3.10] were shown, they would be two parallel straight lines.) The log of the relative potency is the horizontal distance between the two curves, in the same way

as for the linear and four-parameter logistic models given for quantitative responses (sections 3.3 *Parallel-Line Models for Quantitative Responses* and 3.4 *Nonlinear Models for Quantitative Responses*).

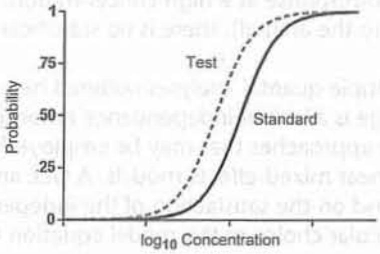


Figure 3.6. Example of Parallel Sigmoid Curves.

Estimating the Model Parameters and Relative Potency—Two methods are available for estimating the parameters of logit and probit models: maximum likelihood and weighted least squares. The difference is not practically important, and the laboratory can accept the choice made by its software. The following assumes a general logistic regression software program. Specialized software should be similar.

Considering the form of equation [3.10], one observes a resemblance to linear regression. There are two independent variables, x = log(z) and T. For each animal, there is a yes/no dependent variable, often coded as 1 for yes or *response* and 0 for *no* or *no response*. Although bioassays are often designed with equal numbers of animals per concentration, that is not a requirement of analysis. Utilizing the parameters estimated by software, which include β_0, β_1, and β_2 and their standard errors, one obtains the estimate of the natural log of the relative potency:

$$\text{Estimate of log of relative potency} = \frac{\hat{\beta}_2}{\hat{\beta}_1}$$

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\beta}_2 / \hat{\beta}_1$. The confidence interval for the relative potency is then [antilog(L), antilog(U)], where [L, U] is the confidence interval for the log relative potency.

Assumptions—Assumptions for quantal models have two parts. The first concerns underlying assumptions related to the probability of response of each animal or unit in the bioassay. These are difficult to verify assumptions that depend on the design of the assay. The second part concerns assumptions for the statistical model for P(z). Most important of these are parallelism and linearity. These assumptions can be checked much as for parallel-line analyses for quantitative responses.

In most cases, quantal analyses assume a standard binomial probability model, a common choice of distribution for dichotomous data. The key assumptions of the binomial are that at a given concentration each animal treated at that concentration has the same probability of responding and the results for any animal are independent from those of all other animals. This basic set of assumptions can be violated in many ways. Foremost among them is the presence of litter effects, where animals from the same litter tend to respond more alike than do animals from different litters. Cage effects, in which the environmental conditions or care rendered to any specific cage makes the animals from that cage more or less likely to respond to experimental treatment, violates the equal-probability and independence assumptions. These assumption violations and others like them (that could be a deliberate design choice) do not preclude the use of logit or probit models. Still, they are indications that a more complex approach to analysis than that presented here may be required (see ⟨1032⟩).

Checking Assumptions—The statistical model for P(z) assumes linearity and parallelism. To assess parallelism, equation [3.10] may be modified as follows:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T + \beta_3 T * \log(z)$$

Here, β_3 is the difference of slopes between Test and Standard and should be sufficiently small. [The T*log(z) term is known as an *interaction term* in statistical terminology.] The measure of nonparallelism may also be expressed in terms of the ratio of slopes, $(\beta_1 + \beta_3)/\beta_1$. For model-based confidence intervals for these measures of nonparallelism, bootstrap or profile likelihood methods are recommended. These methods are not covered in this general chapter.

To assess linearity, it is good practice to start with a graphical examination. In accordance with equation [3.10], this would be a plot of log[(y + 0.5)/(n − y + 0.5)] against log(concentration), where y is the total number of responses at the concentration and n is the number of animals at that concentration. (The 0.5 corrections improve the properties of this calculation as an estimate of log[P/(1 − P)].) The lines for Test and Standard should be parallel straight lines as for the linear model in quantitative assays. If the relationship is monotonic but does not appear to be linear, then the model in [3.10] can be extended with other terms. For example, a quadratic term in log(concentration) could be added: $[\log(\text{concentration})]^2$. If concentration needs to be transformed to something other than log concentration, then the quantal model analogue of slope-ratio assays is an option. The latter is possible but sufficiently unusual that it will not be discussed further in this general chapter.

Outliers—Assessment of outliers is more difficult for quantal assays than for quantitative assays. Because the assay response can be only *yes* or *no*, no individual response can be unusual. What may appear to fall into the outlier category is a single response at a low concentration or a single no-response at a high concentration. Assuming that there has been no cause found (e.g., failure to properly administer the drug to the animal), there is no statistical basis for distinguishing an outlier from a rare event.

Alternative Methods—Alternatives to the simple quantal analyses outlined here may be acceptable, depending on the nature of the analytical challenge. One such challenge is a lack of independence among experimental units, as may be seen in litter effects in animal assays. Some of the possible approaches that may be employed are Generalized Estimating Equations (GEE), generalized linear models, and generalized linear mixed-effects models. A GEE analysis will yield standard errors and confidence intervals whose validity does not depend on the satisfaction of the independence assumption.

There are also methods that make no particular choice of the model equation for the sigmoid. A commonly seen example is the Spearman–Kärber method.

4. CONFIDENCE INTERVALS

A report of an assay result should include a measure of the uncertainty of that result. This is often a standard error or a confidence interval. An interval (c, d), where c is the lower confidence limit and d is the upper confidence limit, is a 95% confidence interval for a parameter (e.g., relative potency) if 95% of such intervals upon repetition of the experiment would include the actual value of the parameter. A confidence interval may be interpreted as indicating values of the parameter that are consistent with the data. This interpretation of a confidence interval requires that various assumptions be satisfied. Assumptions also need to be satisfied when the width or half width [(d-c)/2] is used in a monograph as a measure of whether there is adequate precision to report a potency. The interval width is sometimes used as a suitability criterion without the confidence interpretation. In such cases the assumptions need not be satisfied.

Confidence intervals can either be *model-based* or *sample-based*. A model-based interval is based on the standard errors for each of the one or more estimates of log relative potency that come from the analysis of a particular statistical model. Model-based intervals should be avoided if sample-based intervals are possible. Model-based intervals require that the statistical model correctly incorporate all the effects and correlations that influence the model's estimate of precision. These include but are not be limited to serial dilution and plate effects. Section 4.3 *Model-Based Methods* describes Fieller's Theorem, a commonly used model-based interval.

Sample-based methods combine independent estimates of log relative potency. Multiple assays may arise because this was determined to be required during development and validation or because the assay procedure fixes a maximum acceptable width of the confidence interval and two or more independent assays may be needed to meet the specified width requirement. Some sample-based methods do not require that the statistical model correctly incorporate all effects and correlations. However, this should not be interpreted as dismissing the value of addressing correlations and other factors that influence within-assay precision. The within-assay precision is used in similarity assessment and is a portion of the variability that is the basis for the sample-based intervals. Thus minimizing within-assay variability to the extent practical is important. Sample-based intervals are covered in section 4.2 *Combining Independent Assays (Sample-Based Confidence Interval Methods).*

4.1 Combining Results from Multiple Assays

In order to mitigate the effects of variability, it is appropriate to replicate independent bioassays and combine their results to obtain a single reportable value. That single reportable value (and not the individual assay results) is then compared to any applicable acceptance criteria. During assay development and validation, analysts should evaluate whether it is useful to combine the results of such assays and, if so, in what way to proceed.

There are two primary questions to address when considering how to combine results from multiple assays:

Are the assays mutually independent?

A set of assays may be regarded as mutually independent when the responses of one do not in any way depend on the distribution of responses of any of the others. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined or the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other assays. Assays on successive days using the original and retained dilutions of the Standard, therefore, are not independent assays. Similarly, if the responses, particularly the potency, depend on other reagents that are shared by assays (e.g., cell preparations), the assays may not be independent.

Assays need not be independent in order for analysts to combine results. However, methods for independent assays are much simpler. Also, combining dependent assay results may require assumptions about the form of the correlation between assay results that may be, at best, difficult to verify. Statistical methods are available for dependent assays, but they are not presented in this general chapter.

Are the results of the assays homogeneous?

Homogeneous results differ only because of random within-assay errors. Any contribution from factors associated with intermediate precision precludes homogeneity of results. Intermediate precision factors are those that vary between assays within a laboratory and can include analyst, equipment, and environmental conditions. There are statistical tests for heter-

ogeneity, but lack of statistically significant heterogeneity is not properly taken as assurance of homogeneity and so no test is recommended. If analysts use a method that assumes homogeneity, homogeneity should be assessed during development, documented during validation, and monitored during ongoing use of the assay.

Additionally, before results from assays can be combined, analysts should consider the scale on which that combination is to be made. In general, the combination should be done on the scale for which the parameter estimates are approximately normally distributed. Thus, for relative potencies based on a parallel-line, parallel-curve, or quantal method, the relative potencies are combined in the logarithm scale.

4.2 Combining Independent Assays (Sample-Based Confidence Interval Methods)

Analysts can use several methods for combining the results of independent assays. A simple method described below (Method 1) assumes a common distribution of relative potencies across the assays and is recommended. A second procedure is provided and may be useful if homogeneity of relative potency across assays can be documented. A third alternative is useful if the assumptions for Methods 1 and 2 are not satisfied. Another alternative, analyzing all assays together using a linear or nonlinear mixed-effects model, is not discussed in this general chapter.

Method 1—Independent Assay Results from a Common Assay Distribution—The following is a simple method that assumes independence of assays. It is assumed that the individual assay results (logarithms of relative potencies) are from a common normal distribution with some nonzero variance. This common distribution assumption requires that all assays to be combined used the same design and laboratory procedures. Implicit is that the relative potencies may differ between the assays. This method thus captures interassay variability in relative potency. Note that the individual relative potencies should not be rounded before combining results.

Let R_i denote the logarithm of the relative potency of the ith assay of N assay results to be combined. To combine the N results, the mean, standard deviation, and standard error of the R_i are calculated in the usual way:

$$\text{Mean } \bar{R} = \sum_{i=1}^{N} R_i / N$$

$$\text{Standard Deviation } S = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(R_i - \bar{R})^2}$$

$$\text{Standard Error } SE = S/\sqrt{N}$$

A 100(1 − α)% confidence interval is then found as

$$\bar{R} \pm t_{N-1,\alpha/2} SE,$$

where $t_{N-1,\alpha/2}$ is the upper α/2 percentage point of a t-distribution with N − 1 degrees of freedom. The quantity $t_{N-1,\alpha/2}SE$ is the expanded uncertainty of $\bar{R}$. The number, N, of assays to be combined is usually small, and hence the value of t is usually large.

Because the results are combined in the logarithm scale, the combined result can be reported in the untransformed scale as a confidence interval for the geometric mean potency, estimated by antilog($\bar{R}$),

$$\text{antilog}\left(\bar{R} - t_{N-1,\alpha/2}SE\right), \text{antilog}\left(\bar{R} \pm t_{N-1,\alpha/2}SE\right)$$

Method 2—Independent Assay Results, Homogeneity Assumed—This method can be used provided the following conditions are fulfilled:

(1) The individual potency estimates form a homogeneous set with regard to the potency being estimated. Note that this means documenting (usually during development and validation) that there are no contributions to between-assay variability from intermediate precision factors. The individual results should appear to be consistent with homogeneity. In particular, differences between them should be consistent with their standard errors.
(2) The potency estimates are derived from independent assays.
(3) The number of degrees of freedom of the individual residual errors is not small. This is required so that the weights are well determined.

When these conditions are not fulfilled, this method cannot be applied and Method 1, Method 3, or some other method should be used. Further note that Method 2 (because it assumes no inter-assay variability) often results in narrower confidence intervals than Method 1, but this is not sufficient justification for using Method 2 absent satisfaction of the conditions listed above.

Calculation of Weighting Coefficients—It is assumed that the results of each of the N assays have been analyzed to give N estimates of log potency with associated confidence limits. For each assay, i, the logarithmic confidence interval for the log potency or log relative potency and a value L_i are obtained by subtracting the lower confidence limit from the upper. (This formula, using the L_i, accommodates asymmetric confidence intervals such as from Fieller's Theorem, section 4.3 *Model-Based Methods*). A weight W_i for each value of the log relative potency, R_i, is calculated as follows, where t_i has the same value as that used in the calculation of confidence limits in the ith assay:

$$W_i = \frac{4t_i^2}{L_i^2} \qquad [4.1]$$

Calculation of the Weighted Mean and Confidence Limits—The products W_iR_i are formed for each assay, and their sum is divided by the total weight for all assays to give the weighted mean log relative potency and its standard error as follows:

$$\text{Mean } \bar{R} = \sum_{i=1}^{N} W_iR_i / \sum_{i=1}^{N} W_i$$

$$\text{Standard Error SE} = 1/\sqrt{\sum_{i=1}^{N} W_i}$$

A 100(1 − α)% confidence interval in the log scale is then found as

$$\bar{R} \pm t_{k,\alpha/2}\text{SE} \qquad [4.2]$$

where $t_{k,\alpha/2}$ is the upper α/2 percentage point of a t-distribution with degrees of freedom, k, equal to the sum of the number of degrees of freedom for the error mean squares in the individual assays. This confidence interval can then be transformed back to the original scale as for Method 1.

Method 3—Independent Assay Results, Common Assay Distribution Not Assumed—Method 3 is an approximate method that may be considered if the conditions for Method 1 (common assay distribution) or Method 2 (homogeneity) are not met.

The observed variation then has two components:

- the intra-assay variation for assay i:

$$S_i^2 = 1/W_i$$

- the inter-assay variation:

$$S_B^2 = \frac{1}{N-1}\sum_{i=1}^{N}(R_i - \bar{R})^2 - \frac{1}{N}\sum_{i=1}^{N} S_i^2$$

For each assay, a weighting coefficient is then calculated as

$$W_i' = \frac{1}{S_i^2 + S_B^2}$$

which replaces W_i in equation [4.1] and where t in equation [4.2] is often approximated by the value 2.

4.3 Model-Based Methods

Many confidence intervals are of the form:

Confidence interval = value ± k times the standard error of that value.

For such cases, as long as the multiplier k can be easily determined (e.g., from a table of the t-distribution), reporting the standard error and the confidence interval are largely equivalent because the confidence interval is then easily determined from the standard error. However, the logarithms of relative potencies for parallel-line models and some parameterizations of nonlinear models and the relative potencies from slope-ratio models are ratios. In such cases, the confidence intervals are not symmetric around the estimated log relative potency or potency, and Fieller's Theorem is needed. For these asymmetric cases the confidence interval should be reported because the standard error by itself does not capture the asymmetry.

Fieller's Theorem is the formula for the confidence interval for a ratio. Let R = a/b be the ratio for which we need a confidence interval. For the estimates of a and b, we have their respective standard errors, SE_a and SE_b, and a covariance between them, denoted Cov. (The covariance is a measure of the degree to which the estimates of a and b are related and is proportional to the correlation between the estimates of a and b.) The covariance may be 0, as for some parameterizations of standard parallel-line analyses, but it need not be. The confidence interval for R then is as follows:

$$(R_L, R_U) = \frac{\left\{\hat{R} - \frac{g\text{Cov}}{SE_b^2} \pm \frac{t}{\hat{b}}\sqrt{(1-g)SE_a^2 + \hat{R}^2SE_b^2 - 2\hat{R}\text{Cov} + \frac{g\text{Cov}^2}{SE_b^2}}\right\}}{1-g}$$

where

$$g = \frac{t^2SE_b^2}{\hat{b}^2}$$

and t is the appropriate t deviate value that will depend on the sample size and confidence level chosen (usually 95%). If $g > 1$, it means that the denominator, $\hat{b}$, is not statistically significantly different from 0 and the use of the ratio is not sensible for those data.

For those cases where the estimates of a and b are statistically uncorrelated (Cov = 0), the confidence interval formula simplifies to

$$(R_L, R_U) = \frac{\left\{\hat{R} \pm \frac{t}{\hat{b}}\sqrt{(1-g)SE_a^2 + \hat{R}^2 SE_b^2}\right\}}{1-g} \qquad [4.3]$$

5. ADDITIONAL SOURCES OF INFORMATION

A variety of statistical methods can be used to analyze bioassay data. This chapter presents several methods, but many other similar methods could also be employed. Additional information and alternative procedures can be found in the references listed below and other sources.

1. Bliss CI. *The Statistics of Bioassay.* New York: Academic Press; 1952.
2. Bliss CI. Analysis of the biological assays in U.S.P. XV. *Drug Stand.* 1956;24:33–67.
3. Böhrer A. One-sided and two-sided critical values for Dixon's outlier test for sample sizes up to n = 30. *Econ Quality Control.* 2008;23:5–13.
4. Brown F, Mire-Sluis A, eds. *The Design and Analysis of Potency Assays for Biotechnology Products.* New York: Karger; 2002.
5. Callahan JD, Sajjadi NC. Testing the null hypothesis for a specified difference—the right way to test for parallelism. *Bioprocessing J.* 2003:2;71–78.
6. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves. *Am J Physiol.* 1978;235:E97–E102.
7. European Directorate for the Quality of Medicines. *European Pharmacopoeia,* Chapter 5.3, Statistical Analysis. Strasburg, France: EDQM; 2004:473–507.
8. Finney DJ. *Probit Analysis.* 3rd ed. Cambridge: Cambridge University Press; 1971.
9. Finney DJ. *Statistical Method in Biological Assay.* 3rd ed. London: Griffin; 1978.
10. Govindarajulu Z. *Statistical Techniques in Bioassay.* 2nd ed. New York: Karger; 2001.
11. Hauck WW, Capen RC, Callahan JD, et al. Assessing parallelism prior to determining relative potency. *PDA J Pharm Sci Technol.* 2005;59:127–137.
12. Hewitt W. *Microbiological Assay for Pharmaceutical Analysis: A Rational Approach.* New York: Interpharm/CRC; 2004.
13. Higgins KM, Davidian M, Chew G, Burge H. The effect of serial dilution error on calibration inference in immunoassay. *Biometrics.* 1998;54:19–32.
14. Hurlbert, SH. Pseudo replication and the design of ecological field experiments. *Ecological Monogr.* 1984;54:187–211.
15. Iglewicz B, Hoaglin DC. *How to Detect and Handle Outliers.* Milwaukee, WI: Quality Press; 1993.
16. Nelder JA, Wedderburn RWM. Generalized linear models. *J Royal Statistical Soc, Series A.* 1972;135:370–384.
17. Rorabacher DB. Statistical treatment for rejection of deviant values: critical values of Dixon's "Q" parameter and related subrange ratios at the 95% confidence level. *Anal Chem.* 1991;63:39–48.

Add the following:

▲⟨1039⟩ CHEMOMETRICS

1. INTRODUCTION
    - 1.1 Scope and Purpose
    - 1.2 Content Summary of Document
    - 1.3 Audience
2. WHAT IS CHEMOMETRICS?
3. MODEL LIFECYCLE
    - 3.1 Model Development: Calibration
    - 3.2 Method Validation
    - 3.3 Model Monitoring
    - 3.4 Model Update and Method Transfer
    - 3.5 Revalidation

General Chapters

4. APPLICATIONS OF CHEMOMETRICS
4.1 Qualitative
4.2 Quantitative
GLOSSARY
APPENDIX
REFERENCES

1. INTRODUCTION

1.1 Scope and Purpose

This chapter provides guidance regarding scientifically sound practices for the chemometric analysis and interpretation of typical multivariate data for compendial and industrial applications. Established chemometric practices, including calibration and validation, for applications using different analytical technologies (e.g., spectroscopic, chromatographic, and others) and for different purposes (e.g., fingerprinting, identification, classification, properties prediction, and others) are discussed under a lifecycle approach. Both qualitative and quantitative applications are described.

The chapter discusses how method quality and performance are ensured through the proper lifecycle management of a chemometrics-based model, including the selection of appropriate algorithms, calibration, validation, verification, transfer, and ongoing maintenance steps.

This chapter may be viewed as a supplement to other guidance chapters such as *Analytical Data—Interpretation and Treatment* ⟨1010⟩, which are mainly concerned with the analysis and interpretation of univariate data.

NOTE—It should not be inferred that the multivariate analysis tools mentioned in this chapter form an exhaustive list. Other equally valid models may be used at the discretion of the manufacturer and other users of this chapter.

1.2 Content Summary of Document

The mind map below (*Figure 1*) provides a visual representation of the content of this chapter. This diagram is meant to assist the reader by showing how the various concepts and practices of chemometrics relate to each other.

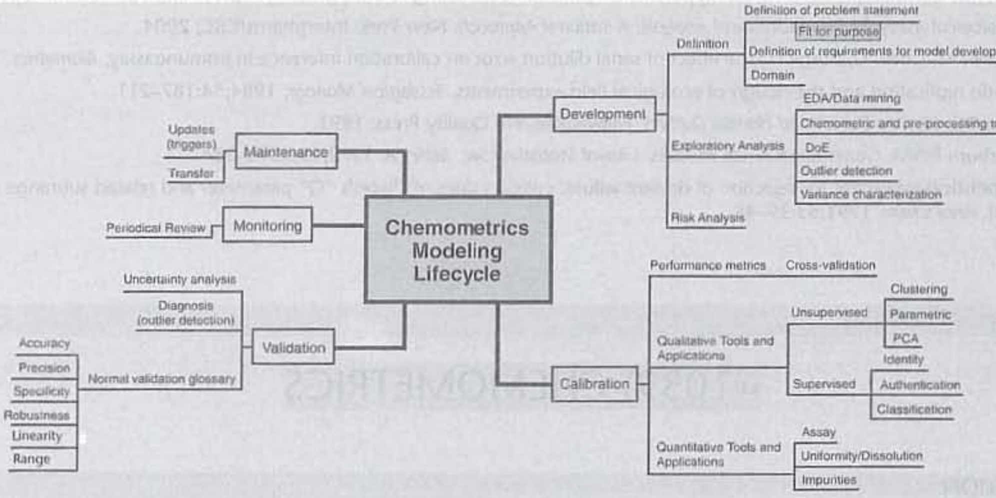


Figure 1. Content summary of document.

1.3 Audience

This chapter provides direction for both the chemometrician applying chemometric techniques to develop a model for a given application and the analyst that runs the model within an analytical procedure. The chemometrician will find support regarding algorithm selection for a given application and guidance for ensuring model performance throughout its lifecycle. The analyst will gain insight regarding the strengths and limitations of the chemometrics techniques as applied to support their applications.

2. WHAT IS CHEMOMETRICS?

Chemometrics was originally defined as the chemical discipline that uses mathematical, statistical, and other methods that employ formal logic to accomplish two objectives: 1) to design or select optimal measurement procedures and experiments, and 2) to provide the maximum amount of relevant chemical information by analyzing chemical data. (*1,2*) More specifically,

"chemometrics" has come to mean the application of multivariate methods for the analysis of chemical or related data, although the algorithms in question may be used to extract information out of almost any measured data, regardless of origin—chemical, physical, biological, pharmaceutical, or others. This chapter does not focus on the development of optimal procedures or methods and the applied design of experiments (DoE), but rather on the analysis of multidimensional data collected from an analytical instrument, such as spectroscopic and chromatographic data. A multivariate data set (i.e., multivariate data obtained for a number of samples or objects) forms an $m \times n$ data table or matrix. The matrix is represented by **X**, with m as the number of samples or objects and n as the number of variables measured for each sample (*Figure 2*).

Consequently, the data analysis techniques considered in this chapter will be multivariate in nature. Depending on the purpose of the data treatment, different tools will be applied. Initially, the data handling techniques can be divided into two categories: unsupervised and supervised. The unsupervised tools use only the **X** matrix to extract information, but in supervised data analysis, in addition to the **X** matrix the samples are also described by a **y** vector. This is an $m \times 1$ table containing property information for each sample (e.g., concentration, enzyme inhibition activity). The supervised data analysis techniques are used to build a model between the **X** matrix and the **y** vector. In chemometric modeling, the equations provided are data driven to empirically describe the underlying variance in the data for either unsupervised or supervised purposes. The different techniques or tools applied for the different purposes are discussed in more detail in *4. Applications of Chemometrics*.

The most commonly used unsupervised technique (principal component analysis, or PCA) and supervised technique (partial least squares regression, or PLS) are by nature latent projection approaches (see *Figure 2*), which transform a large number of potentially correlated **X** variables, such as intensities at different retention times or wavelengths, into a possibly smaller number of uncorrelated variables (principal components, or PCs; latent variables). As can be seen in *Figure 2*, the original n-dimensional space was transformed to a two-PC space. When samples from the original data space are projected onto this lower-dimensionality space, the resulting sample coordinates are called scores (**T**). Visualization of the scores along two or more PCs forms a score plot that contains information on the relationships among different samples. The loadings (**P**) are a linear combination of the original variables and the coefficients or weights used in this linear combination. The loadings for specific latent variables also can be plotted in what is called a loading plot. The loading plot contains information on the relative importance among the original variables. Both scores and loading plots enable visualization and understanding of the underlying data structure (i.e., the presence of groups/clusters and/or outliers) within a reduced dimensional space. The variable matrix (**E**) produced by the model is defined as the residual error. The sample information along axes of common variance is captured by the model's PCs. Variance unaccounted for by these PCs (residual error) is left for each sample at each variable, forming the residual matrix (**E**).

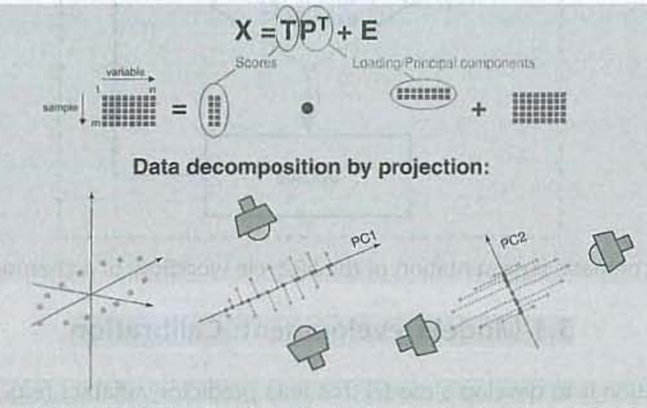


Figure 2. Schematic representation of latent projection techniques.

3. MODEL LIFECYCLE

The development of a chemometric model, as part of an analytical procedure, aims to fulfill a predefined, intended purpose. The intended purpose is typically a statement describing what to measure, the output format, and the level of performance needed with the result, and should be in accordance with the analytical target profile (ATP). The ATP may specify performance criteria for key characteristics of the analytical procedure output and/or decision risk probabilities expected in routine use of the analytical procedure.

Calibration of the model encompasses an iterative process that involves selection of the sample set to be used to develop the chemometric model, tuning of an appropriate chemometric algorithm with the necessary preprocessing algorithm, and evaluation of model performance according to predefined metrics for the ATP. During the validation stage, the method performance is demonstrated to fulfill the intended purpose and ATP. Both the knowledge gained during calibration and the assessment of specific metrics and corresponding limits are used to evaluate performance and define a method maintenance protocol that will be used for the monitoring stage, before deployment to routine use. Changes that may have an impact on model performance should trigger a defined set of activities to update it. Model update may also be triggered by the necessity of performing method transfer, in the course of its lifecycle, to different equipment or another site. The extent of the revalidation will be defined by the magnitude of the model update. *Figure 3* shows a schematic representation of the described workflow.

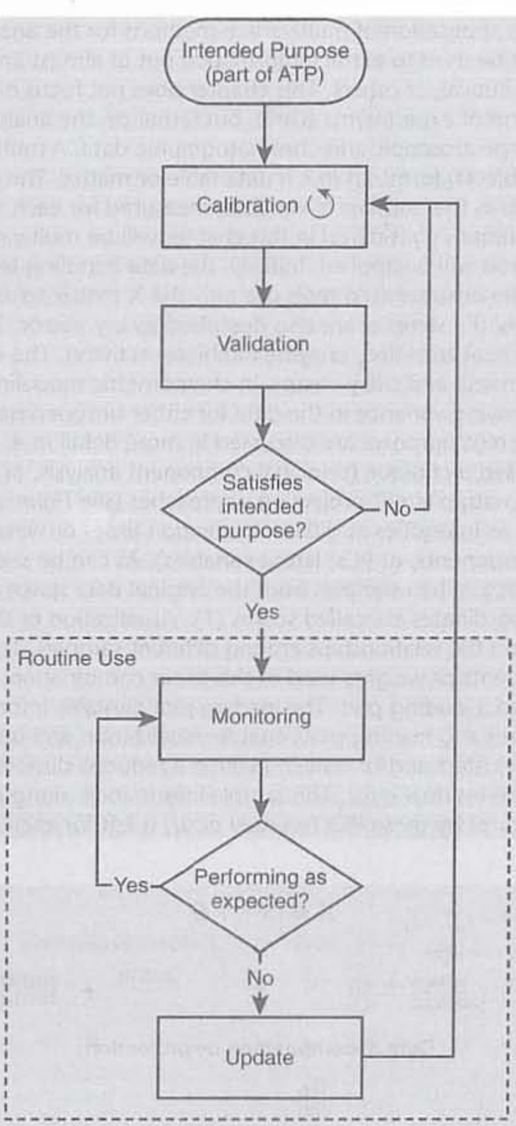


Figure 3. Schematic representation of the lifecycle workflow of a chemometric model.

3.1 Model Development: Calibration

The goal of multivariate calibration is to develop a model that links predictor variables (e.g., absorbance values for the range of spectral wavelengths) with response variables such as concentrations of certain chemical compounds. Response variables are typically more difficult or costly to obtain than predictor variables. Calibration is a critical step because details of the final model are influenced by many factors including the selection of samples, variables, an algorithm, and preprocessing options, as well as the quality of the reference method initially used (where applicable) to measure the response variable(s). Thus, the order of individual components of model calibration presented in this section does not always represent the order of operation, and individual steps might be repeated in an iterative fashion depending upon the outcome of subsequent steps. To perform well, the calibration model should be robust to intended variations.

SAMPLE SELECTION

Selection of samples for the calibration model is a critical step in model development. Scientifically sound rationales must be used to select representative calibration samples with respect to two criteria: the type/range of variability, and the number of samples. The range of response values in selected samples should cover the entire expected range of response variation for the intended application. The range and type of variation in predictors may include relevant variability in factors other than the property being predicted, such as particle size, different lots of ingredients used for sample preparation, analyst-to-analyst and day-to-day variation, and other sources of variation. In the manufacturing setting, batches of samples representing within and outside specification should be included. A risk-based approach is recommended for deciding which factors and variability will

be included in the calibration samples. Performing a failure mode and effect analysis (FMEA) or a feasibility study to evaluate the effects of such factors is one of the commonly used approaches.

The required number of samples increases as the complexity of the calibration model (i.e., the type and the range of variability in both response and predictor variables) increases. In general, the larger the number of samples, the higher the probability that correct results can be achieved throughout the range of calibration. Meanwhile, the distribution of the calibration samples also deserves attention. A uniform distribution of the samples is preferred, although it is not required in all cases, depending on the specifics of the application. Scientifically sound rationales must be in place to justify the number and the distribution of calibration samples. When obtaining new calibration samples becomes costly, the DoE and/or historical database approaches are commonly used alternatives to build calibration models.

Data obtained from selected samples (i.e., a selected number of rows of the **X** matrix in *Figure 2*) should undergo further triage. Exploratory data analysis (see *Qualitative Application Examples*) may be valuable for understanding data structure, detecting potential clusters or groups of samples, identifying outliers, and selecting representative samples. Outliers may be detected using metrics that describe how well a given observation fits within model space (e.g., Hotelling's T^2 for latent variable methods) and distance to the model subspace (e.g., residual for latent variable methods).

PREPROCESSING

The goal of preprocessing is to deploy mathematical transformations on the column of the **X** matrix (*Figure 2*) to amplify variation of interest within the data set and attenuate extraneous variation for both variables and observations. Preprocessing is an important initial step for most data analyses, and can be applied iteratively with variable selection. The initial selection of preprocessing options should be guided by an understanding of the sample data and/or the underlying analytical technique. Guidance may also be obtained through exploratory multivariate data analyses (see *Qualitative Application Examples*). Initial selections can be modified or refined within the context of subsequent model performance optimization. This process is almost always cyclic in practice; comparison across different preprocessing strategies leads to a better understanding of the data, which can further refine the preprocessing to maximize the signal-to-noise ratio.

However, there are two points of caution. Preprocessing should be used judiciously because: 1) overprocessing can attenuate the signal and inflate undesirable variations; and 2) many chemometric methods can accommodate noisy and visually unappealing data. Furthermore, in some instances the preprocessing has been accomplished on the instrument; many algorithms used by instrument vendors are proprietary, and it may be impossible to know exactly what modifications have been made to the data. The chemometrician must be aware of, and cautious about, any preprocessing already applied in the reported raw data.

Preprocessing may consist of transformation, normalization, and/or other mathematical treatment of data. Preprocessing of samples (rows of data matrix) may include mean or median centering, scaling, and other procedures. Variables (columns of data matrix) can also be transformed, aligned (e.g., time warping), or scaled (e.g., autoscaling). Mean centering is probably the most common preprocessing method. For example, the use of mean-centered data in the regression model removes the intercept term, resulting in a potentially parsimonious model. It is also common to remove the linear baseline (e.g., bias/offset correction) or polynomial interferences or to apply digital filtering techniques such as derivatives, wavelets, Fourier filter, or a Savitzky-Golay filter to remove or attenuate linear offsets and noise contribution. Normalization is the scaling of observations, giving objects similar impact on model creation by: 1) accounting for factors other than the analyte of interest that impact measurement (e.g., sample path length variability caused by particle size, sample volume, or tablet thickness); 2) correcting for variation in concentration or mass of an analyte for qualitative models; and 3) attenuating measurement artifacts (e.g., instrument performance drift). When variables are highly correlated, such as in spectroscopic measurements, normalization is typically performed via standard normal variate (SNV) and multiplicative scatter correction (MSC). These normalizations are typically conducted on each sample (row).

In cases where variables have different units, sensitivities, or signal-to-noise ratios, variable scaling is typically applied to correct for that; autoscaling is typically used, but the analyst may consider other alternatives, such as using the pooled standard deviations of measurement replicates as scaling/weight parameters. These normalizations are typically conducted on each variable (column). No guidelines exist for deciding which preprocessing approach is the best for a given data set. It is often a trial-and-error process as the analyst applies different preprocessing techniques to find the best one. Combinations of preprocessing techniques can be applied to a given data set as well.

ALGORITHM SELECTION

Many different algorithms have been tried with varying degrees of success in chemometrics applications. The methods include relatively simple ones such as multivariate linear regression and its modifications (robust or weighted regression); the widely used latent variables approaches such as principal components regression (PCR) and PLS; local methods such as k-nearest neighbors (kNN); and the more sophisticated methods such as support vector machines (SVM) and artificial neural networks. More details on commonly used algorithmic tools can be found in *4. Applications of Chemometrics*. In general, it is difficult to predict which algorithm will produce the best results for a particular data set. The multitude of choices for sample selection, variable selection, and data normalization and preprocessing—as well as the combination of tuning parameters for each algorithm—could significantly affect the performance of the calibration model. Sometimes, the choice of algorithm even iter-

ates with the steps of sample selection, variable selection, and preprocessing. Thus, the choice of algorithm may depend on the task at hand, software availability, and the subject matter expert's familiarity with the method. Another consideration is that some algorithms provide useful tools for diagnostics and interpretation—such as PLS scores, loadings, and coefficients plots, whereas others are sometimes referred to as "black boxes" because their inner workings can be difficult to interpret (e.g., neural networks).

It is important to keep in mind that many algorithms are empirical in nature. Almost always, some kind of model can be developed. Thus, the results should be evaluated critically to ensure that results generated by the model are relevant and correct, and that model performance meets the requirements of the ATP. This can be accomplished by cross-validation, and ultimately, by validation with the independent test data set that was not used to develop the model.

VARIABLE SELECTION

The intended purpose of variable selection is to identify a subset of predictor variables (i.e., the column of the X matrix in *Figure 2*) that can improve the performance of the model. The underlying rationale for variable selection in chemometrics is twofold. First, certain predictor variables could be irrelevant to the intended purpose, and ideally, these variables should be minimized or removed from the model. For example, only specific wavelengths of the whole range generated by the spectrometer may bear information relevant to the variation in levels of the response variable. Variable selection for this purpose should be based on first principles and experience. Inclusion of unrelated predictors, such as irrelevant spectral regions, could potentially degrade performance of the model. A smaller number of variables from the preprocessed data can be used to achieve superior performance, such as accuracy, precision, and robustness.

The second part of the rationale for variable selection is to avoid overfitting. Overfitting is the situation where the model is not only describing the intended variability in the data but also includes the modeling of the noise. The latter has a negative influence on the predictive properties of the model. Typically in chemometrics, the number of predictor variables (hundreds or thousands) is larger than the number of observations (dozens to hundreds). There are two general strategies for handling the issue of overfitting. One strategy is to manually or computationally select only a subset of predictor variables and use them for model development. Predictor variables can be selected manually (e.g., choosing certain spectral wavelengths characteristic of a given compound) or by using a variety of statistical selection methods such as stepwise regression and using simple univariate statistical measures such as the *F*-test, *t*-test, correlation coefficient, signal-to-noise ratios, intensity thresholds, variable importance in projection metric, or genetic algorithms. Multivariate approaches may use PC variable loadings, linear discriminants, or regression coefficients to define the key features.

In the second strategy for avoiding overfitting, variables are not selected at all. The latent variable methods—PLS and PCR—by their nature have the capability to selectively give more weight to the important predictor variables and de-weight the less important ones. These new latent variables, or components, are constructed as linear combinations of the observed variables. If combined with variable selection, the performance of latent variable models could potentially be improved, because some predictor variables contain only noise, thus perturbing the model-building process.

Selecting a subset of the predictor variables that are informative will reduce the size of the data set, provide computational efficiency, and obtain better models for postprocessing. One caveat for applications such as authentication is that eliminating predictor variables that are devoid of information may prevent the recognition of adulterants or novel objects that have features in these excluded predictor variables.

CROSS-VALIDATION

In practice, cross-validation is used to obtain an estimate of the model performance and to fine-tune an algorithm's parameters (e.g., the number of components for PLS). This is accomplished by repetitive splitting of the data (i.e., the number of rows of the X matrix in *Figure 2*) into a calibration set, which is used to develop the model, and a testing set (or internal validation set), which is used to make predictions and to compare actual and predicted values.

The approach of n-fold cross-validation is commonly used to split the data into n subsets to perform cross-validation. In each of the n iterations, n–1 subsets are used to develop the model, which is used to predict the remaining n^{th} data split. The procedure is repeated until all subsets are predicted. The error between reference values and predicted values during cross-validation is then recorded as root-mean-squared error of cross-validation (RMSECV). Multiple approaches exist for splitting the data into n segments. Regardless of how one decides to split the original calibration data, one aspect that deserves attention is that any batches used in calibration and cross-validation must not be considered or reused as an independent dataset for method validation. The most straightforward form is called leave-one-out cross-validation (LOOCV), where samples are removed one at a time. The LOOCV can involve intensive computations; its results can be heavily affected by outliers and are less consistent than the results of other forms of cross-validation. It is also possible to split the data according to prior information, such as one subset per batch given the available historical data set across multiple batches. Another option for cross-validation is bootstrapping, where in each iteration a certain proportion of data is randomly sampled to create a calibration set, while the remainder of the data are used as a testing set. The procedure is repeated many times (typically 100 or more cycles) to obtain a stable estimate of prediction error.

The measure of error is the most common figure of merit used to characterize model performance during cross-validation. The intended purpose of the method determines the nature of the errors, such as the misclassification rate for qualitative

methods and prediction error for quantitative methods. Irrespective of qualitative or quantitative applications, two metrics are commonly used to characterize error within cross-validation: root-mean-squared error of calibration (RMSEC) and RMSECV. The RMSEC is calculated for the samples when left in the calibration, which monotonically decreases with each additional factor (i.e., PC) added into the model. In comparison, the RMSECV that is calculated during cross-validation will decrease until the last meaningful (i.e., relevant signal-containing) factor is added. Then, as each additional factor is incorporated into the model, the RMSECV will increase, indicating that the calibration data are being overfit. The plot of the RMSEC and/or the RMSECV versus the number of factors in the model is referred to as a predicted residual error sum-of-squares plot. In general, the best practice is to avoid inclusion of factors beyond where the minimum of the RMSECV plot line occurs. In addition, correlation between observed and predicted values (e.g., R^2) is also commonly used to assess performance of quantitative methods. Finally, the cross-validating results are only meaningful when the calibration and testing sets are comparable (i.e., drawn from the same population). Otherwise, extrapolation may lead to incorrect predictions.

3.2 Method Validation

The objective of validation is to demonstrate that the performance of a method is suitable for its intended purpose. Validation of a model and validation of a method are two different activities. Model validation routinely involves the use of an internal validation set or cross-validation to assess the appropriate parameters of a model via identification or quantification error and uncertainty. Parameters often include the range of variables, the type of preprocessing, the model rank, the choice of the algorithm, and others. These activities have been addressed in detail in *3.1 Model Development: Calibration*. In comparison, method validation must be based upon a fully independent external validation set and must follow the validation requirement described in *Validation of Compendial Procedures* ⟨1225⟩, according to the method type category. The acceptance criteria should be justified for the intended purpose. During the lifecycle, method revalidation is necessary after a model transfer or model update. The method validation and revalidation strategy should be risk- and science-based and appropriate to its impact level.

The typical performance characteristics for method validation are specificity, accuracy, precision, linearity, range, and robustness for quantitative models, and specificity and robustness for qualitative models. The metrics and their descriptions are discussed below. In addition to those typical performance metrics, metrics such as limit of detection (LOD), limit of quantitation (LOQ), sensitivity, analytical sensitivity, and effective resolution may not be required for validation purposes but could be useful for understanding the boundary of the method performance for a specific analytical application and the analytical technique that a model is associated with.

PERFORMANCE CHARACTERISTICS FOR METHOD VALIDATION

The sections that follow provide method validation attribute descriptions and metrics.

Accuracy: Statistical comparison between predicted and reference values is recommended. For quantitative applications, root mean-squared error of prediction (RMSEP), squared error of prediction (SEP), and bias are the typical measures of method accuracy. For qualitative applications such as classification, sometimes misclassification rate or positive prediction rate could be used to characterize method accuracy. To ensure that a model is accurate enough when tested with an independent test set, RMSEP is often found to be comparable to RMSEC and RMSECV and to meet the requirements of ATP.

Precision: The routine metrics of RMSEP and SEP encompass both accuracy and precision. Assessment of precision could involve a determination of the uncertainty associated with the reportable result and a variance component analysis that determines an "error budget" that quantifies the sources of variation that contribute to the uncertainty. For an estimate of precision alone, the standard deviation across results for replicate and independent analyses on the same sample within method, across days, across analysts, across instruments, and across laboratories could be used.

Specificity: For both qualitative and quantitative methods, whenever possible the underlying chemical or physical meaning of the chemometric model should be demonstrated and validated. For example, the scientific meaning of the variables (such as spectral range) used for model construction, data preprocessing, regression vectors, and loading vectors [for PLS, PCR, PLS discriminant analysis (PLS-DA), and PCA] should be demonstrated. Specificity could also be validated by the tendency of sample components (matrix or other nonanalyte compounds present in the sample) to adversely affect the ability of the chemometric method to report results accurately and/or precisely. The level of specificity may be assessed by accuracy and precision in the presence of varying amounts of potentially interfering substances. Substances to be tested can be identified by considering the underlying physical/chemical methodology and modeling approach.

Specificity may be evaluated by including adulterated, substandard, or nonauthentic samples. Authentic samples, both within and out of the target criteria, may not be available for practical or economic reasons. For example, it may not be possible, due to cost, to obtain out-of-target samples for a controlled, large-scale process. Where possible, an exclusion panel of out-of-target samples that may be closely related to the target samples should be considered to increase the confidence in the specificity of the measurement. Simulated off-target samples (such as small-scale samples) may be used to validate the procedure's suitability for the intended purpose and range. In all cases, the suitability of validation samples must be justified with appropriate inclusion and exclusion criteria.

For qualitative methods, the method should demonstrate the capability to correctly identify or classify the samples. The receiver operating characteristic (ROC) curve and/or the probability of identification (POI) are commonly used metrics (detailed

information on ROC curves can be found in *4.1 Qualitative* and *Figure 6*). The ROC approach is intended to illustrate true-positive rate (TPR) and false-positive rate (FPR) over a range of decision thresholds. A good identification method should generate a ROC curve with the area under the curve (AUC) close to 1. For most well-designed identification methods, the probability of a positive identification is near zero when the analyte is not present, and the probability should approach 1 as the analyte concentration or mass increases.

Linearity: The algorithm used for chemometric method construction can be linear or nonlinear, as long as it is appropriate for modeling the relationship between the analytical signal and the analyte. The measures commonly used to assess either the model fit or its predictive properties are the correlation coefficient, slope, *y*-intercept, and residual sum of squares of the plot between the predicted versus observed results. Note that the plot between the residual versus observed results across the analytical range is expected to show no pattern.

Range: The range of a method should be appropriate for its intended use (e.g., specification).

Robustness: Typical factors to be considered include the normal variability of materials (e.g., lot-to-lot variability), operating environment variability, instrument variability, such as minor instrument maintenance, and method parameter variability, such as the number of spectra averaged in a spectrometer. In conjunction with the validation results, the method development strategy—such as the design of the calibration set or library and the choice of model parameters—can be taken into account to demonstrate the method robustness.

VALIDATION SAMPLES

Other general aspects to be considered for chemometric method validation include the validation samples. The validation samples should be independent of the calibration samples to demonstrate the ability of the model to predict. Being independent means that the validation samples were not used in the calibration set or used for model optimization. Internal validation or cross-validation is typically used during calibration for model parameter optimization, but is not considered sufficient for final method validation. The validation samples should be selected based upon the ATP and the desired performance characteristics of the model. Method robustness is based on the evaluation of authentic samples with typical sources of variance. For pharmaceutical or dietary supplement products, validation samples of nominal production scale, such as those routinely manufactured for in-process, release, and/or stability testing, may be included. For botanical articles, taxonomic identity, geographic origin, season of collection, and other variants may be included. For naturally sourced materials such as many food ingredients and excipients, variables such as the geographic or microbiological source of the material, processing conditions, impurity composition, and other relevant attributes may be included in the validation sample set.

ACCEPTANCE AND DIAGNOSTIC CRITERIA

As with any other analytical procedure, the acceptance criteria for a chemometric method should be defined before execution of the validation protocol. If the chemometric model was developed using data from a secondary technique [e.g., near-infrared (NIR) or Raman] with reference values from a primary analytical procedure [e.g., gravimetric, nuclear magnetic resonance (NMR) spectroscopy, or high-performance liquid chromatography (HPLC)], the ATP for validation may not exceed that which is obtainable by the primary method. Some exceptions may occur, for example, it may be possible to achieve superior precision using a secondary procedure, although the accuracy will be limited to that of the reference technique.

In addition to those attributes addressed in *Performance Characteristics for Method Validation*, method validation must also take into consideration the setting of diagnostics limits for a multivariate model before deployment for routine use. Samples that are out of model space are considered outliers and not suitable for obtaining a reportable model prediction. The model diagnostic should have a demonstrated capability to flag any of the out-of-model-space samples. To set up the diagnostics limits, two cases must be considered. The first is to determine the statistical distribution of leverage and residual within-the-calibration data set. The second is to prepare intended in- and out-of-model-space validation samples to test the limit. For instance, for an NIR spectroscopy-based content uniformity method, the in- versus out-of-model-space samples could be samples at target label claim versus samples containing active pharmaceutical ingredient (API) concentrations outside of the intended range for the method.

3.3 Model Monitoring

Throughout the model lifecycle, changes that can affect model performance may occur. Procedures should be in place for continuous performance verification of the model and for model update and procedure revalidation if necessary. A specific order among model monitoring, model update, and model transfer does not exist. Scientifically sound rationales must be applied to determine an appropriate sequence for the intended application.

A control strategy for checking model performance over its lifecycle should be developed and documented as part of model development and procedure validation. The strategy should identify the necessary elements for ongoing monitoring and evaluation of model performance. In addition, a plan for monitoring, analyzing, and adjusting the model should be in place with a measurement frequency that allows identification of excursions related to critical aspects of the model. The level of maintenance should be considered part of the risk assessment activities and should be adequate for the model criticality. If applicable,

analytical instrumentation used to generate the inputs for the model should be qualified and also subjected to a continuous verification plan (for relevant guidance, see general chapters related to the applicable analytical instrumentation).

Ongoing assurance of performance of the model throughout its lifecycle should include:

- Ongoing monitoring and review of the model
- Evaluation of risk assessment
- Evaluation of post-implementation changes and predefined model maintenance
- Model update and procedure revalidation as needed

The ongoing review of a model should occur at predefined intervals and also should be triggered by events that may have an impact on model performance. Examples of such events include changes in raw materials variability or manufacturer; changes in the upstream process that may alter the sample matrix (e.g., process equipment or operation settings); drifts in model prediction; and out-of-specification (OOS) or out-of-trend (OOT) results given by the model (dependent upon the root cause of the OOS or OOT). In addition to triggers that are based on the prediction output of the model, triggers based on model diagnostics metrics and corresponding action rules should be included. Multivariate models may be more strongly affected by aberrant data signatures than are univariate models. Special care is needed to: 1) justify the multivariate model diagnostics statistically, 2) verify with data from the model development and procedure validation process, and 3) implement multivariate diagnostics for monitoring as part of the control strategy. Comparison of model predictions and reference or orthogonal procedures should take place on a periodic basis or as part of the investigation triggered by the review process.

The use of model diagnostics when applied to a new sample ensures that the model prediction is valid with regard to the calibration and validation sets used during model development and procedure validation, and also ensures that the result does not constitute an outlier. The observation of an outlier means that the result is invalid, but it is not a reliable indication of an OOS result; an OOS result is an observation produced by a model when the prediction falls outside the acceptance criteria and the model diagnostics are within the thresholds. In the case of qualitative models, nonconforming results should be treated as outliers and should trigger an investigation; the output of such an investigation will indicate whether the result is OOS.

The review process for a model should produce a decision regarding the need for an extension of model maintenance; such a decision may be the result of a risk-assessment exercise or the outcome of a predefined decision workflow. Model criticality and usage will define the extension of model maintenance, which can include restraining the model conditions; adjusting the calibration set (samples can be added, replaced, or removed); or even completely rebuilding the model. The decision, and corresponding rationale, must be scientifically sound and documented.

3.4 Model Update and Method Transfer

Model updating must be considered part of the analytical procedure verification, and both the justification and the activities must be documented as a part of the analytical procedure lifecycle. Before performing a model update, it is critical to understand the underlying causal factor that has prompted the update. The reasons for model updating can be roughly divided into two categories.

The first category is when the calibration set simply needs to be expanded. In this case, nothing has actually changed in terms of the response of the instrument to specific analytes. Instead, the original model is no longer valid because of the expanded range of original calibration, the addition of new analytes, or the occurrence of other, previously unseen variations (e.g., changes in particle size distribution, or the drift of a chemical process to a new steady state). Thus, the calibration space must be expanded with samples exhibiting this variation.

The second category is when the samples are the same but the measurement system response function has changed. This is often due to changes in the measurement components (new light source, clouding of optics, wavelength registration shift) or can be due to method transfer across different instruments. This, in essence, is an instrument standardization problem. Changes in instrument or measurement procedures over time can render a calibration model unusable, in which case a model update becomes necessary or a new model should be developed.

In practice, there are multiple model updating techniques that could be applied to each category of model update. Some updating techniques are relatively straightforward and simple to implement, whereas others are technically complex. Selection of an appropriate model updating approach must be based on a full understanding of the underlying causal factor. As a general rule, simple updating methods (e.g., slope and bias adjustment, as described below) should be considered first.

Before any model maintenance work is initiated, it is good practice to confirm that the fundamental construct of the original model—such as data preprocessing and variable selection—remains sound. For both qualitative and quantitative applications-based model updates, a straightforward preprocessing approach and a scientifically sound variable selection approach are recommended as the initial attempt to address challenges associated with updating the model. These approaches are equally applicable to differences caused by instruments, measurement conditions, or sample matrices.

SLOPE AND BIAS ADJUSTMENT

One of the simplest model-updating methods is to postprocess the predictions with a bias adjustment, a slope adjustment, or both. This approach is often used for quantitative applications. For some qualitative applications, this approach may also be useful, depending on the nature of the method. In a limited set of circumstances, bias/slope adjustments are expected to work. For example, if the matrix of the samples being predicted were systematically different from the calibration/validation

sample set, the model predictions would be in error by a constant value. Bias/slope adjustments, however, would not correct for any new variation in the data, such as variation that would result from a different matrix (e.g., new analytes/interferents). Therefore, applying slope/bias adjustments without a full understanding of the underlying causal factor is not recommended. Guidance may be obtained via inspection of residuals corresponding to the new data obtained using the existing model or, alternately, from supplemental exploratory data analyses using techniques such as PCA (see *4. Applications of Chemometrics*).

CALIBRATION EXPANSION

When expanding the original calibration set, one should consider the number of new samples to be added, the impact (leverage) of the added samples on the overall composition of the new calibration set, and how to partition any new samples between the calibration and validation sets. It may be appropriate to simply augment the original calibration and validation sets with all the new samples, or it may be advisable to use a subset of the new and original samples. Multiple approaches, such as the Kennard-Stone algorithm and nearest-neighbor approach, are available to aid in the selection of new samples to add to an existing calibration set, but in general, all the approaches use methods to identify new samples on the basis of high leverage (i.e., model influence). Hotelling's T^2, multivariate score plots, and their corresponding limits are also effective approaches for accomplishing this goal.

CALIBRATION TRANSFER

Instrument standardization and calibration transfer methods are used to transform the response function of a measurement system so that it matches that of a reference measurement system. The reference measurement system could consist of a completely different analyzer, or it could be the same analyzer before it experienced a response-function shift. The vast majority of these methods generally requires the use of stable transfer samples that are measured on the original instrument and require the instrument to be standardized at the same time. In addition, the approaches commonly used for instrument standardization could also be applied effectively to address the challenges resulting from changes in sample matrix and measurement conditions.

3.5 Revalidation

Before the redeployment of a multivariate model, appropriate procedure revalidation should be established using criteria equivalent to those used in the original validation protocol. This revalidation is necessary to document the validity of the model as part of the analytical procedure verification. The nature and extent of the revalidation procedure, including aspects such as scientific justification and experimental approaches, must be based on the cause of the update and the nature of the corrective action required for establishing suitable performance. Revalidation should be documented as part of the analytical procedure lifecycle.

4. APPLICATIONS OF CHEMOMETRICS

As discussed in *2. What is Chemometrics?*, chemometric analyses may be performed in either a supervised or unsupervised manner depending on the availability of data and the specifics of a given application. This section provides an explanation of these different analysis scenarios, as well as the chemometric tools (i.e., algorithms) that are commonly used. Additionally, several specific applications will be described in detail.

4.1 Qualitative

GENERAL ASPECTS

Qualitative chemometric analyses may be performed by using supervised and/or unsupervised approaches. However, to be incorporated into analytical procedures that are alternatives to compendial methods, the performance of any chemometric model must be verified as described in *3. Model Lifecycle*. This may not be possible if very little is known about the samples being analyzed. Nevertheless, unsupervised analyses play an important supporting role during the development of chemometric alternatives to compendial procedures within the lifecycle framework, and are recommended for use before the development of subsequent supervised approaches (examples are provided in *Qualitative Application Examples*).

Qualitative compendial procedures are those that seek to supply the value of some categorical descriptor for an unknown sample. Examples of categorical properties include (among others):

- Chemical identity: microcrystalline cellulose versus API
- Morphology: polymorph A versus B; monoclinic versus triclinic
- Sample authenticity: authentic versus nonauthentic and/or adulterated
- Sample origin: facility A versus B; lot ABC-001 versus lot XYZ-002

Categorical properties can be modeled effectively using supervised chemometric algorithms that leverage either proximity or distance in multivariate space, resulting in a qualitative assignment of samples to one or more classes depending on the application and the technique used. According to ⟨1225⟩, procedures based on models of this type are suitable for incorporation into Category IV methods for identification and should be validated to demonstrate adequate specificity for the intended use. "Identification" is a term that is generally used to describe a range of analysis scenarios; several common ones are described in *Qualitative Tools.*

Supervised techniques used for classification purposes, besides the analytical sensor information, make use of discrete information related to the samples in the data set (e.g., class labels). It is intended to define the borders of the clusters (or classes), providing statistical criteria, in addition to the visualization techniques (e.g., scores plot of PCs from a PCA). The borders then can be used as acceptance criteria for inclusion or exclusion of new samples into a given class. Several useful tools are available to the analyst for building the model, such as linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA), soft independent modeling of class analogy (SIMCA), kNN, and PLS-DA. More complex techniques also may be used with justification. Each type of model has its own strengths and weaknesses. If a particular algorithm does not give the desired level of performance, a different algorithm may be selected. These aspects are discussed further in *Qualitative Tools.*

For model development, optimization, and validation, the classification and discrimination techniques follow the same process described in *3. Model Lifecycle.* These techniques develop a threshold or limits to make class assignments. The simplest form of classification is a two-class system (e.g., good/bad or A/B). When a new sample is classified as good or bad (A or B) by the model, this results from what is called a discrimination technique. Classification is the technique of assigning a new sample as A, B, either A or B, or neither A nor B. The error rate of the model is the total number of incorrect classifications divided by the total number of samples. Depending on the requirements of the application and the associated risk levels, the error rate(s) might be class-specific or pooled across classes. The specific details of the application will determine whether the assignment of a sample to more than one class would be an acceptable result. Where this must be avoided, classification thresholds should be set appropriately during model development to guarantee single-class results.

QUALITATIVE TOOLS

PCA: PCA is a commonly used exploratory analysis tool that was briefly introduced in *2. What is Chemometrics?.* PCA is a variable reduction technique and acts on the data by defining new variables, so-called principal components (PCs, see *Figure 2*). PCs are orthogonal and are defined in the direction of the largest (remaining) variance in the data. The results of PCA are discussed in terms of PC scores and loadings, which may be plotted graphically in two (and sometimes three) dimensions for visualizing clusters of samples or outlying samples (score plots, see *Figure 4*), whereas the loadings plots provide information on the original variables. The scores are the projections of the samples on the PCs, whereas the loadings provide the weights (coefficients) by which the values of the original variables are multiplied to generate the component score for a particular sample. Loadings are rich in information regarding which variables in the matrix are prominent and can be used to "understand" the information captured by the latent variables that provide the basis for the observed sample distribution in the scores plot (*Figure 4*). The PCs may also be used as the basis for quantitative models via PCR (see *4.2 Quantitative*).

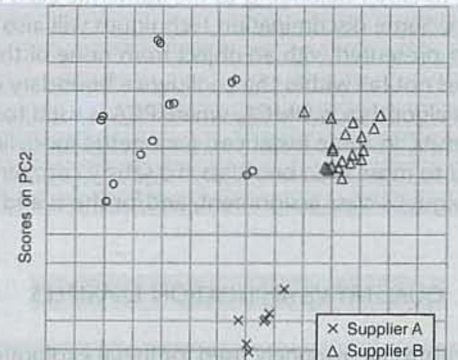


Figure 4. PCA scores plot: projection of PC1 versus PC2.

Clustering algorithms: The aim of clustering analyses is to partition data into different groups. Some methods, such as hierarchical cluster analysis (HCA), result in tree-like structures called dendrograms (*Figure 5*) that provide information on groups of samples or outlying objects occurring in the data set. Dendrograms can be built in many different ways and thus may show different aspects of the data set. A first possibility, called divisive or top-down methods, starts from the entire data set, which is split in consecutive steps until each object is in an individual cluster or all elements of a cluster fulfill a given similarity criterion. In the second scenario, called agglomerative or bottom-up methods, the opposite is done. Starting from individual objects/

clusters, those most similar are merged until everything is in one cluster. Of the two options, bottom-up approaches tend to be computationally less intensive, are part of most computer packages, and are more frequently used.

The parameters used to express (dis)similarity between objects or clusters (see *y*-axis on *Figure 5*) can be either correlation-based (e.g., correlation coefficient, *r* for similarity or 1−|*r*| for dissimilarity), distance-based (e.g., Euclidian distance) measures, or a combination of both (e.g., Mahalanobis distance). Two clusters are linked in the dendrogram at the height related to this (dis)similarity measure. The parameter applied is expressed in such a way that clusters/objects linked low (i.e., close to the *x*-axis) are similar, whereas those linked high are dissimilar. Many different methods or criteria exist for use in deciding which objects/clusters are consecutively merged [e.g., single linkage, complete linkage, (weighted) average linkage, and centroid linkage]. Depending on the applied criterion, the dendrograms may look very different. Drawing arbitrary horizontal lines (see *Figure 5*) splits the data set into different groups, which occasionally may be linked to sample properties. Outlying samples are linked very high in the dendrograms, most often as one-object clusters. The stability of clustering results can be affected by many factors, such as noise in the data, sample size, choice of algorithm, distance measure, and others. Divisive methods tend to produce more stable results than agglomerative methods, even though they are hardly ever used.

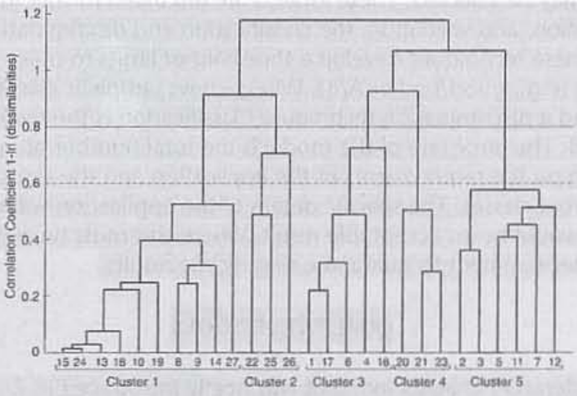


Figure 5. Hierarchical clustering based dendrogram for 27 objects. Abscissa: object numbers.

Parametric modeling: Authentication of a material or formulated product for quality assurance purposes may be achieved via multivariate parametric modeling of the chemical profile (e.g., spectrum or chromatogram). These methods are sometimes referred to as "one-class classifiers". Basically, a parametric model is developed statistically or empirically with a confidence boundary around the data from known authentic samples. Parametric models describe the behavior or structure of the majority of the data from a single class, assuming that a distribution (typically, a multivariate normal one) exists in those data. Predictions against a parametric model identify deviating points outside the significant boundaries of that distribution. Whereas classification or discrimination approaches provide identification (as previously described) of the unknown sample, parametric models will designate the unknown sample as either belonging to the authentic class or as an outlier, or exhibiting features other than those defined by the distribution. Some discrimination techniques will also optimize class separation, and may have greater selectivity, but will tend to fail when presented with an object from none of the classes. In contrast, parametric modeling methods will reject any sample that does not fall within the confidence boundary established for the model. Perhaps the most commonly used parametric modeling algorithm is SIMCA, where PCA is used to model the data and Hotelling's T^2 and Q residuals (DmodX) are used to define the limits. In some instances, parametric modeling approaches will result in "soft classifications" wherein a sample may be assigned to more than one class. To satisfy compendial usage requirements, appropriate action must be predefined for cases of ambiguous class assignment, and/or the α and β error rates must be established and justified according to risk.

QUALITATIVE APPLICATION EXAMPLES

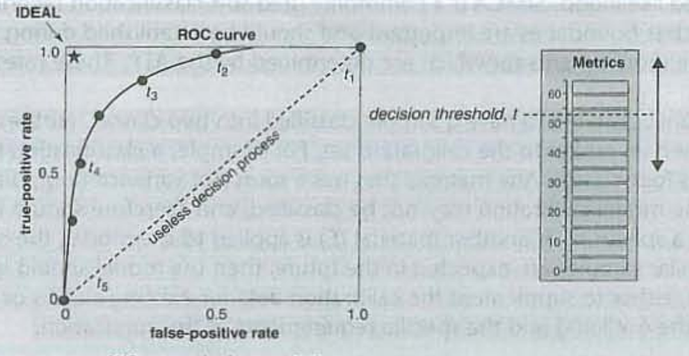
Exploratory analysis: Unsupervised algorithms are routinely used for initial exploratory data analyses, which precede formal calibration and validation exercises. Unsupervised data exploration can rapidly indicate, in the absence of any prior knowledge, whether distinct classes or outliers exist within the pool of available data. For instance, in *Figure 4* a graphical example is shown in which PCA applied to spectroscopic data was used to differentiate between samples from different suppliers. Exploratory models yield latent variables that may be inspected to identify the original variables with the greatest amounts of relevant analyte signal to carry forward into subsequent models or to guide and/or verify the results of variable selection approaches. This might occur during the analysis phase of a DoE study to assess which factors to include in subsequent designs and/or calibrations. Further, exploratory analyses may be used to test and empirically optimize various signal preprocessing options to maximize the relative contribution of desired analyte signal versus signals from sources of interference (e.g., scattering effects, optical path length differences, sample physical properties, and instrumental drift).

Material identity testing: Identification testing is routinely performed for all pharmaceutical active ingredients, excipients, drug products, and packaging materials to authenticate them and verify that the correct materials are used in manufacturing, release testing, and packaging operations. *Spectrophotometric Identification Tests* ⟨197⟩ illustrates one of the most widely used

analytical techniques for identification, Fourier transform IR spectroscopy. Typically, identification testing involves comparing the sample spectrum to the spectrum of a reference sample and assessing the level of agreement between the two. Different chemometric algorithms can be applied for identification, and the general model development and maintenance should follow the guidance in *3. Model Lifecycle*. All methods for identity testing are supervised classification methods because either a single reference spectrum or a set of reference spectra from material(s) with known identity is utilized by these algorithms. Algorithms can be applied in either original variable space or transformed variable space (such as latent variable space) after data preprocessing.

According to 〈1225〉, models of this type are suitable for incorporation into Category IV methods for identification and should, at a minimum, be validated to demonstrate adequate specificity for the intended use. An example approach for performance verification is the documentation of TPR and FPR. TPR is the percentage of samples that are correctly identified, whereas FPR is the percentage of samples that are incorrectly identified. TPR is also referred to as sensitivity, and FPR is referred to as specificity. Regardless of which approach is taken, there is always a trade-off between FPR and TPR, and this trade-off behavior and overall procedure performance are best visualized in a ROC curve plot (see *Figure 6*).

A classical ROC curve is generated by plotting the TPR on the ordinate and the FPR on the abscissa. Each point on a ROC curve represents a specific TPR and FPR pair obtained at a specific threshold. The threshold is typically a number generated by the algorithm, and when it is exceeded, this corresponds to a positive identification. This threshold value is determined during the procedure development process. The threshold could be the *P* value from hypothesis testing, hit quality index (HQI) threshold, Mahalanobis distance threshold, PLS discriminant score threshold, or some other threshold.



AUC: area under the curve (0-1)

Figure 6. ROC curve plot showing the AUC (*3*).

During procedure development, a number of known positive and negative samples are examined, and the algorithm output for each sample is recorded. The performance of the method when operating at a particular threshold *t* is characterized by a single TPR/FPR pair, and the full operating characteristic curve can be produced by plotting TPR/FPR pairs over a range of decision thresholds. A false-positive identification corresponds to a decision threshold that is too low. False positives can be minimized by raising the decision threshold. However, excessively high decision thresholds can produce a false-negative identification.

A good identification method should generate a ROC curve that produces one or more TPR/FPR pairs in the top-left corner of the plot, as shown in *Figure 6*. This characteristic is best captured by the AUC. A random decision method is represented by the diagonal line with an AUC of 0.5, whereas a good identification method should have an AUC closer to 1. The shape of ROC curve depends upon a combination of spectral properties of the sample and the chemometric algorithm used for identification. Characterization of the ROC curve is a fundamental task associated with method development and validation.

For a two-class model, false positives and false negatives are the incorrectly assigned samples. Together with the true positives and true negatives, they provide the basis for the sensitivity and specificity of the model. The analyst may wish to care about one type of error (α or β) more than another, and therefore could change the model to be more conservative when choosing a threshold. Thresholds can be used as action criteria or evaluation criteria.

Complex material authentication testing: Nutraceuticals and samples of herbal origin may be complex mixtures of numerous components. Authentication relies upon assessment of the presence of multiple analytes and their relative concentrations as well as potentially demonstrating the absence of adulterants and/or related materials. Thus, authentication in this scenario is a multivariate decision process that is appropriate for chemometric approaches. Typically, a characteristic "fingerprint" pattern incorporating information from the various analytes (and adulterants, potentially) that are present in the sample is obtained by applying a combination of separation and characterization techniques or spectroscopic measurements. The chemical composition information in the sample fingerprint provides a basis for multivariate pattern recognition. In particular, techniques with exceptional analytical selectivity, such as mass spectrometry and NMR spectroscopy, have emerged as powerful fingerprinting tools, especially when operated as detectors for chromatographic separations of multicomponent samples. Chromatographic fingerprinting is recognized as a viable identification procedure for herbal medicines by both the World Health Organization and the U.S. Food and Drug Administration. (*4,5*)

Depending on the origin and purity of the sample, it may be appropriate to develop a multivariate classification model using either the entire fingerprint or a selected subset of peaks. Variable selection approaches may be useful in determining the opti-

mal approach, depending on the nature of the samples and/or the ATP. It is crucial that the samples have an associated class label or quantitative property (activity) obtained by an objective reference technique (separate from that used to generate the fingerprint) to enable supervised modeling and subsequent model performance verification. Care should be taken to ensure that appropriate sources and levels of variability are included in model development and performance verification exercises based on the intended use of the test.

Several useful tools are available to the analyst for building the model, such as LDA and QDA, SIMCA, kNN, and PLS-DA. More complex techniques may also be used with justification (e.g., SVM).

Chemometric methods for multicomponent sample authentication are consistent with *Validation of Compendial Procedures* ⟨1225⟩, *Validation, Data Elements Required for Validation, Category IV*. Various numerical methods may be used to verify predictive performance. As discussed in *Qualitative Tools* for pure material identification, predictive performance may be verified by comparison of the TPR and FPR once a classification threshold appropriate to the application has been established. It is crucial to finalize this threshold before formal validation of the overall analytical method.

Classification: This is a supervised learning technique. The goal of calibration is to construct a classification rule based on a calibration set where both **X** and **Y** (class labels) are known. Once obtained, the classification rule can then be used for the prediction of new objects whose **X** data are available.

Classification models may be divided into two main groups: hard and soft. Hard classification directly targets the classification decision boundary without producing a probability estimation. Examples of hard classification techniques are kNN and expert systems. Soft classification estimates the class conditional likelihood explicitly and then makes the class assignment based on the largest estimated likelihood. SIMCA is a commonly used soft classification technique. In either scenario, the thresholds to determine the class boundaries are important and should be established during method development based on the resulting TPR and FPR, the requirements for which are determined by the ATP. These rates should also be verified as part of the full procedure validation.

In many applications, it is unacceptable to have a sample classified into two classes. Further, if a sample is not classified into any class, the sample is deemed an outlier to the calibration set. For example, a classification model built from NIR spectra of four materials (A, B, C, D) has four classes. Any material that has a source of variance (e.g., different water content) not previously seen and included in the model calibration may not be classified, and therefore should be identified as an outlier and investigated. Similarly, when a spectrum of another material (E) is applied to the model, the model should not be able to classify it into a given class. If similar samples are expected in the future, then the model should be revised to include the outlier samples in the calibration set, either to supplement the calibration data for existing classes or to create an entirely new class, depending on the nature of the outlier(s) and the specific requirements of the application.

4.2 Quantitative

GENERAL ASPECTS

When the intended use of an analytical procedure is the determination of one or more sample components or properties that have values that can vary continuously rather than categorically, then a quantitative model is required. Similar to the process for qualitative modeling, quantitative chemometric models are produced in a supervised manner using both the independent **X**-block and dependent **y** variables to ensure optimal predictive performance. The difference arises in the nature of the **y** values, which are continuous/numerical (rather than categorical) for quantitative applications. Per ⟨1225⟩, quantitative chemometric models are suitable for incorporation into Category I, II, or III methods for determination of the content of drug substance or finished drug product, impurity levels, or performance characteristics, respectively. In-process tests applied in an offline manner or an in/on/at-line manner that produce the value for a continuous variable would also represent appropriate uses of quantitative chemometric models. The specific requirements of the analysis per the ATP (see *3. Model Lifecycle*) should be used to guide the validation metrics used to demonstrate performance.

QUANTITATIVE TOOLS

The algorithmic tools needed for quantitative applications are wide ranging; for more complete descriptions see *Appendix, Additional Sources of Information*. Tools that are commonly used are briefly described here. As is the case with any chemometric model, the tool must be matched to the ATP, and vice versa. Certain advantages and disadvantages are characteristic of each tool, and the chemometrician must be mindful of these when justifying their use.

Multiple Linear Regression (MLR): This tool establishes a correlation between a dependent variable (or y, response variable) and multiple independent variables (or x, explanatory variables) by fitting a linear equation to observed data. MLR has two broad applications, as follows.

The first broad application of MLR is to establish a model that explains the importance or magnitude of effect on the response variable, and this is typically used in the DoE screening or response analysis. Typically the input variables are mutually independent and by design represent large magnitudes. Often, the higher order of terms such as square terms and interaction terms are included, and a step-wise procedure is used to exclude those that make a smaller contribution to the model. Caution is advised to avoid having too many variables in the model, because including too many terms will overfit the model. The adjusted R^2 statistic is typically used to estimate whether there are too many factors in the model, although other metrics may

also be appropriate. Adjusted R^2 is a modification of the standard coefficient of determination that takes into account the number of model terms and the number of samples, resulting in a plot of R^2 (adjusted) that has a maximum value beyond which additional terms result in overfitting.

The second broad application of MLR is to establish a prediction model for analytical purpose. MLR can be used to fit a predictive model to an observed data set of **y** and **x** values. The established model can be used to predict the value **y** from the **x** of new samples. The model for prediction purpose created from correlated **x** inputs is suitable to explore the relationship between **y** and individual **x**'s.

The prediction MLR model can be used in a procedure or method for a pharmaceutical compendial test. For example, a filter NIR, which is designed for a specific use such as water testing in the product, produces a "spectrum" of only a few data points.

The development and validation can follow the same procedures as are used with latent variable models such as a PLS. During the development and validation of an MLR model: 1) appropriate data preprocessing needs to be used; 2) cross-validation is used to estimate the model performance; and 3) an independent data set is used to assess the model.

A limitation of MLR is that model predictive performance may be negatively impacted when the modeled variables are collinear. The issue of collinearity is not unique to MLR. It is mitigated in PLS modeling via generation of a new basis set of orthogonal (noncollinear) latent variables that are linear combinations of the original variables. However, this is not relevant for MLR models. In MLR models, variable selection or transformation (e.g., mean centering) may be needed to avoid or reduce collinearity in the calibration set. Even though MLR models have some diagnostic metrics to identify outliers and highly leveraged samples in the calibration, they do not have diagnostic metrics (such as Hotelling's T^2 and **X** residual test) that PLS models offer during the prediction of new samples.

PCR: This is a two-step modeling approach in which a PCA is first performed on the **X**-block data to obtain a basis set of PCs. The PCs are then used in combination with the **y** data to develop a regression model to predict the **y** values.

PLS: This is one of the most commonly used chemometric algorithms for both quantitative and qualitative (PLS-DA, see *Qualitative Tools*) modeling scenarios. The computational advantage that PLS offers over MLR and PCR is that the PCs are derived in the PLS algorithm using the **X** and **y** data simultaneously. This often results in predictive models that require fewer latent variables compared to PCR, thereby improving robustness. PLS is capable of handling mildly nonlinear relationships, whereas PCR and MLR are linear modeling techniques. Further, more than one **y** variable can be modeled using the same **X**-block data via different algorithmic approaches. PLS-1 results in a separate model for each **y** variable. PLS-2 will produce a single model capable of simultaneously predicting the values of two or more **y** variables. The relative advantages of each approach may vary from one application to another. For instance, if different mathematical preprocessing is necessary to resolve each component to be predicted, separate PLS-1 models may provide improved performance over a single PLS-2 model.

QUANTITATIVE APPLICATION EXAMPLES

Pharmaceutical dosage form assay and/or uniformity of content: Assay of the API content in the final dosage form is commonly performed via measurement of a sample drawn from a homogenous composite of a number of individual dosage units (e.g., tablets or capsules). The quantitative result is then reported as an "average content" (expressed as percentage of target API amount) for the batch tested. Uniformity of content may be tested using a method similar to the assay, but measurements are performed on individual samples. The reported result is primarily based on the variability in assay results across the individual dosage units analyzed. Assay and uniformity of content methods are intended to characterize the "major" component in the sample(s) in question and are considered Category I tests per the definitions established in 〈1225〉. In the majority of cases, the target analyte will be the active ingredient in the sample, although quantitative assays for other components exist throughout the compendia as well.

For pharmaceutical solid dosage forms, the majority of assay procedures are based upon HPLC. HPLC analysis is time consuming and often involves the use of large volumes of solvent for the mobile phase. However, HPLC is highly linear and is typically calibrated using univariate mathematics (i.e., one measured variable is directly and uniquely proportional to sample concentration).

In contrast, alternative methods for assay that are based on spectroscopy (e.g., NIR and transmission Raman spectroscopies) offer the advantage of increased analysis speed and are nondestructive. However, in many cases the analyte signal may not display the same degree of linearity or signal-to-noise as with the corresponding HPLC method. Moreover, no single variable is directly and uniquely related to the concentration of interest. For this reason, multivariate models, often based on PLS regression, are commonly used in spectroscopic procedures for assay and uniformity of content.

For Category I tests, it is crucial to demonstrate the accuracy, precision, specificity, linearity, and range of results during the validation of the overall analytical procedure. Each of these performance aspects involves considerations during multivariate model development or validation that are distinct from those related to univariate techniques.

Most spectroscopic methods are not quantitative in an absolute sense. For example, models developed using NIR or Raman data must be calibrated relative to a primary reference technique (often HPLC or NMR spectroscopy). Given the nondestructive nature of NIR and Raman, it is straightforward to analyze a given set of samples using one of these techniques and then subsequently analyze the same set of samples via HPLC or NMR spectroscopy to obtain the reference content values. This approach is typical for assays of solid dosage forms, however, it is worth noting that reference values for assays of some dosage forms, APIs, polymorphs, or excipients might be derived from other techniques such as gravimetric data recorded during the

General Chapters

calibration sample preparation. The chemometric models are developed via a multivariate calibration using the NIR or Raman spectral data and the content values from the reference technique. Accuracy is determined by using the absolute difference between the model predictions and the reference values. Precision is determined by using the standard deviation of procedure results alone, such as from replicate analyses of the same sample. Thus, precision is a measure of performance for the analytical procedure as a whole. The model itself will be absolutely precise. That is, given the same spectral input, the same numeric output will always be generated. That said, precision may be influenced by the specifics of the chemometric modeling approach (e.g., preprocessing, modeling algorithm, number of latent variables) and therefore precision should be evaluated during model development as well as during validation. It is important to note that the accuracy of chemometric models will not be able to exceed the accuracy of the reference analytical technique used to calibrate the model. However, it may in certain cases be possible to exceed the precision of the reference technique.

The range of the method will be determined by the calibration samples that are used to develop the multivariate model. According to ⟨1225⟩, it is recommended that assay calibration samples should have analyte contents ranging from 80%–120% of the target amount. For evaluating uniformity of content, the recommended range is 70%–130% of the target amount. In some cases, both the assay results and uniformity results may be obtained from the same set of sample measurements. Averaging of a requisite number of individual dosage-unit assay results will provide a value equivalent to the "average content" parameter. Calculation of the "acceptance value" (see *Uniformity of Dosage Units* ⟨905⟩) using this mean result combined with the standard deviation for the same set of individual dosage unit results will provide a uniformity of content result. In this scenario, a single calibration set that spans the wider of the two recommended ranges (70%–130%) should be used.

If it is not practical to obtain a sufficiently wide range of content values in calibration samples produced at commercial manufacturing scale, calibration samples may also be produced in a smaller scale or in the laboratory. In this scenario, all attempts should be made to replicate the physical properties representative of the commercial scale. Additionally, care should be taken to verify that minimal bias exists between samples from different scales. Commercial-scale samples may need to be incorporated into the model (and/or procedure) validation process to verify the accuracy and precision of the model (and/or procedure) results against any differences and/or variability in physical properties. Mathematical processing algorithms (e.g., normalization, second derivatives) should be optimized as much as possible during initial exploratory data analysis (see *Qualitative Application Examples*) to mitigate prediction bias resulting from these factors. For instance, it is known that thickness, particle size, and density differences can lead to spectral slope changes and baseline offsets in NIR spectra. Depending on which specific effect is present, improperly selected preprocessing may not fully correct for spectral differences, leading to errors in prediction.

Fit and predictive performance of chemometric models should be demonstrated via the linearity of a plot of the model outputs versus the reference or nominal values of the target analyte(s), ideally using results from a set of independent test samples. The raw analytical signal may demonstrate a nonlinear relationship with the reference analyte concentrations. Likewise, multiple latent variables may be utilized for the chemometric model building. However, the key aspect of the model that must be demonstrated to vary linearly with analyte concentration is the model outputs, not the inputs. A plot of the prediction residuals versus concentration may assist in revealing any systematic lack of fit. Any observations of patterns in this residuals plot may indicate a need for revision of either 1) the number of latent variables included in the model, 2) the preprocessing mathematics, or 3) the algorithm type used for modeling.

Demonstration of the specificity of the procedure for assay may be based on likely sources of interference or material substitution based on an understanding of the material properties of the sample components and the manufacturing process and/or supply chain. One approach might be to verify that low assay results that are OOS are consistently obtained for a placebo version of the dosage unit. Another approach might be to verify that dosage units of a different product (especially one manufactured in the same facility and/or tested in the same laboratory) result in assay values that are consistently OOS.

Impurity limit tests: Routine testing is required for determination of impurities and/or degradants in intermediates, bulk drug substances, and finished pharmaceutical products. Testing for this purpose may take the form of a limit test (Category II, ⟨1225⟩).

Typically, these impurities tests are based upon HPLC or other methods (e.g., Karl Fischer titration for water). These analytical procedures are highly linear and are typically calibrated using univariate mathematics. However, these methods are often time consuming and involve manual sampling, which is disadvantageous, especially for products having high potency or for those with toxic intermediates produced during API synthesis.

In contrast, alternative methods for assay based on spectroscopy (e.g., NIR and Raman spectroscopy) offer the advantage of increased analysis speed, and more importantly, total elimination of human sampling due to the noninvasive nature of the measurement. However, these advantages are typically offset by decreased sensitivity (LOD) and potentially nonlinear sensor response across the required analyte concentration range as the corresponding reference method. For this reason, multivariate approaches combining spectral preprocessing and latent variable models are often employed in spectroscopic methods for these limit tests. (*6*) However, due to the potential challenges involved in achieving adequate sensitivity for a spectroscopy-based limit test, careful development and feasibility studies must be employed.

For a limit test in Category II procedures, a calibration set containing varied concentrations of the analyte of interest is often used to characterize the performance of such an analytical procedure. Although it is not required, these performance metrics could include accuracy, range, linearity, and others. The use of the performance metrics depends on the nature of the limit test.

In contrast, it is critical to illustrate specificity and LOD during validation of a limit test according to ⟨1225⟩. For specificity, a comparison between loading/regression vector and a spectrum representative of the pure component of interest is often used. Additionally, measuring matrix effects on the determination of the analyte within the specified range is another useful approach to demonstrate that the analytical procedure is specific to the analyte of interest without impacts from other variables introduced in the calibration set. Moreover, evidence of method specificity can be demonstrated by calculation of a selectivity ratio from the calibration data set, defined as the variance of model-reconstructed spectra divided by that of residual spectra. The selectivity ratio at the analyte absorption band is expected to be higher relative to other spectral regions.

Regarding LOD, there is no generally accepted estimator for PLS models. A common practice to determine LOD involves calculating a spectral signal-to-noise ratio. The model results for replicate analyses of a blank sample(s) may be used to represent the noise. Alternatively, the noise may be approximated using the standard error of a multivariate model. Other more elaborate approaches were suggested to address the multivariate nature of PLS models. (*7*) With any approach, the estimated LOD should be verified experimentally using samples with analyte levels at or near that concentration. Because the LOD obtainable via a spectroscopic method will likely be higher than that obtainable via a chromatographic approach, it becomes crucial to demonstrate that the LOD based on the chemometric model meets the requirements of the ATP.

Dissolution testing: In vitro dissolution is a crucial performance test for solid dosage forms that is used to predict in vivo drug-release profiles during drug development and to assess batch-to-batch consistency for quality control purposes. Chemometric models can be used to predict the drug product dissolution from relevant measured product properties, and the method that uses such a model may be established as an alternative procedure. Alternatively, the dissolution profile can be modeled via several responses from different time points on the dissolution curve using individual models.

Dissolution data may be collected as a profile (i.e., a series of values obtained at various time points), even though the acceptance criteria may rely on a single time point. When building a chemometric model, it is essential for the model to be able to predict the entire profile. The number, and spacing in time, of points in the dissolution profile will vary depending upon the product type (e.g., immediate versus modified/extended release). A common approach is to transform the profile into a single value, which then serves as a dependent variable (or **y**, response variable). This variable, combined with another measured variable, can be used to restore the dissolution profile. The transformation can be accomplished by fitting the dissolution profile data into a mechanistic model (such as a first-order rate equation or its variants) or an empirical model (such as a Weibull function). After transformation, a few variables (typically, two or three variables) can be used to represent the dissolution profile. Generally, the two variables are a dissolution rate factor and a plateau factor; a third variable such as lag time may be necessary. The dissolution rate factor, which represents the rate of dissolution, will be the dependent variable for the modeling. The plateau factor represents the final amount of the drug in the solution, which should be equal to the drug content for most products. These two factors are used to restore the profile. Therefore, the model for dissolution prediction is used to predict the dissolution rate.

The key to designing a successful model is defining the input variables. The plateau factor may be obtained from drug content data, whereas the dissolution rate factor may be modeled from a data set of chemical and physical attributes of the product or intermediate product. The necessary knowledge of physicochemical properties and engineering concepts should be used to identify and justify the relevant inputs that have potential impact on the dissolution of the product. This typically involves risk assessment and an additional data collection step such as a small-scale DoE study. The inputs typically include the measured material attributes at various processing stages (such as particle size data for granules or blend; NIR measurements for blend or tablets; and physical tablet properties).

There are two approaches for defining samples in the model calibration: batch samples and individual unit dose samples. For the batch-sample approach, one product batch is treated as a sample, and the input variables are the attribute means of the batch. The individual unit dose approach is to measure the attributes of individual unit doses (such as NIR measurement on the individual tablets). The input variables from both approaches may be supplemented with other raw material properties as justified by the critical quality attributes.

The validation of the model-based dissolution prediction method could be different from the typical HPLC dissolution method validation, depending on the specific approach used. The HPLC dissolution method puts more emphasis on precision, as the dissolution is treated as a Category III method in ⟨1225⟩.

In the batch-sample approach, the model-based method treats dissolution as a batch property instead of a property of individual tablets, and accuracy is the focus of method validation or verification. For example, each batch forms a sample, and it is not realistic to have a large validation sample set. This approach generates a single profile for a batch, and the result at a certain time point can be used to assess the product batch quality. Evaluation of variability must be aligned with the criteria in *Dissolution* ⟨711⟩. The variability of the final dissolution result can be evaluated by analyzing the variations of inputs. Possible ways to evaluate the variation of the sample (batch) include the analysis of variation of inputs, simulations, and dividing a batch into multiple sub-batches.

In the individual unit dose approach, the model-based method evaluates individual dosage units. If many tablets are tested, this becomes a large *n* situation in which the acceptance criteria in ⟨711⟩ should be used with caution. The chemometrician may propose acceptance criteria as long as these criteria are demonstrated (by simulation or other means) to have equivalent or tighter passing criteria than those in ⟨711⟩. In the method validation or verification, it is desirable to have at least one sample that shows low dissolution (near or below the specification), and the chemometric model-based method should demonstrate the capability to distinguish this low-dissolution sample from normal samples.

GLOSSARY

Many common chemometric terms have been defined within the text of this chapter. Some additional terms are defined here and serve as a reference to their usage in the text. For a more complete description, consult the texts listed in *Appendix, Additional Sources of Information.*

Calibration model: A mathematical expression used to relate the response from an analytical instrument to the properties of samples, or to capture the underlying structure of a calibration data set.

Calibration set: Collection of data used to develop a chemometric classification or model.

Derivatives: The change in intensity with respect to the measurement variable (i.e., abscissa). Derivatives are useful for removing baseline offsets (constant or linear) due to sample properties, or for highlighting small changes in a signal, helping to enhance selectivity and sensitivity (e.g., one spectroscopy and chromatographic peak with a shoulder peak adjacent).

Internal validation: The application of resampling statistics such as cross-validation. Subsets of the calibration data set are subjected to a variety of statistical processes to identify which calibration model best fits the available data. Each model is characterized by a statistical parameter. For cross-validation, the entire data set of samples is split into individual samples or groups of samples, which are removed individually from the rest of the samples and tested as unknowns against a calibration model constructed using the rest of the samples. The characteristic statistic is the standard error of cross validation (SECV).

Matrix: A two-dimensional data structure comprised of columns and rows used to organize inputs or outputs during chemometric analyses. Common practice is for each row to correspond to an individual sample and each column to correspond to an individual variable.

Validation set: The data set that challenges the performance attributes of the model. The validation data set is independent of the training data set, although testing on the training data set gives an optimistic view of performance and thus allows for iteration of preprocessing and model tweaking until this optimistic view meets the expectations. Validation or cross-validation is then necessary to adequately gauge the performance of the model. The model performance with the validation data set will always be equal to or worse than its performance with the training data set. If the performance attributes are not met, then the chemometrician must assess whether the model underfits or overfits the data and whether iterations of the model are needed to meet the acceptable error rate. If these efforts fail, it is recommended to back up one step, alter the preprocessing conditions, and perform the same task again.

General Chapters

APPENDIX

Additional Sources of Information

Many books have been written on the subject of chemometrics and multivariate analysis. Various terms not found in the glossary may be found in this short list of additional sources of information.

- Massart DL, Vandeginste BGM, Buydens LMC, De Jong P, Lewi PJ, Smeyers-Verbeke J, eds. *Handbook of Chemometrics and Qualimetrics: Part A.* 1st ed. Amsterdam: Elsevier Science B.V.; 1997.
- Vandeginste BGM, Rutan SC, eds. *Handbook of Chemometrics and Qualimetrics: Part B.* 1st ed. Amsterdam: Elsevier Science B.V.; 1998.
- Mocak J. Chemometrics in medicine and pharmacy. *Nova Biotechnologica et Chimica.* 2012;11(1):11–25.
- Singh I, Juneja P, Kaur B, Kumar P. Pharmaceutical applications of chemometric techniques. *ISRN Anal Chem.* 2013; article ID 795178. http://www.hindawi.com/journals/isrn/2013/795178/. Accessed 25 May 2016.
- Brown S, Tauler R, Walczak B, eds. *Comprehensive Chemometrics: Chemical and Biochemical Data Analysis.* 1st ed. Amsterdam: Elsevier Science B.V.; 2009.
- Varmuza K, Filzmoser P. *Introduction to Multivariate Statistical Analysis in Chemometrics.* Boca Raton, FL: CRC Press (Taylor & Francis Group); 2009.

REFERENCES

1. Massart DL, Vandeginste BGM, Buydens LMC, De Jong P, Lewi PJ, Smeyers-Verbeke J, eds. *Handbook of Chemometrics and Qualimetrics: Part A.* 1st ed. Amsterdam: Elsevier Science B.V.; 1997.
2. Massart DL, Vandeginste BGM, Deming SM, Michotte Y, Kaufman L. *Chemometrics: A Textbook.* 1st ed. Amsterdam: Elsevier Science B.V.; 1988.
3. Brown CD, Davis HT. Receiver operating characteristics curves and related decision measures: a tutorial. *Chemometr Intell Lab Syst.* 2006;80:24–38.
4. World Health Organization. General guidelines for methodologies on research and evaluation of traditional medicine. Geneva, Switzerland: World Health Organization; April 2000. http://apps.who.int/iris/bitstream/10665/66783/1/WHO_EDM_TRM_2000.1.pdf. Accessed 25 May 2016.
5. Food and Drug Administration. Guidance for industry: botanical drug products. Rockville, MD: Food and Drug Administration; June 2004. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070491.pdf. Accessed 25 May 2016.

6. Lambertus G, Shi Z, Forbes R, Kramer TT, Doherty S, Hermiller J, et al. On-line application of near-infrared spectroscopy for monitoring water levels in parts per million in a manufacturing-scale distillation process. *Appl Spectrosc.* 2014;68(4): 445–457.
7. Allegrini F, Olivieri AC. IUPAC-consistent approach to the limit of detection in partial least-squares calibration. *Anal Chem.* 2014;86:7858–7866.

▲*USP40*

⟨1041⟩ BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.

Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only upon approval by the Center for Biologics Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see *Safety Tests—Biologicals* under *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Sterility Tests* ⟨71⟩, *Water Determination* ⟨921⟩, and *Pyrogen Test* ⟨151⟩, as well as *Bacterial Endotoxins Test* ⟨85⟩). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. (See also *Units of Potency* in the *General Notices*.)

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of *USP Supplements* as promptly as practicable.

Vehicles and Added Substances—Vehicles and added substances suitable for biologics are those named in the Food and Drug Regulations.

Containers for Injections—Containers for biologics intended to be administered by injection meet the requirements for *Packaging and Storage Requirements* ⟨659⟩, *Injection Packaging*.

Container Content—The volumes in containers of biologics intended to be administered by injection meet the requirements for *Container Content for Injections* ⟨697⟩.

Labeling—Biologics intended to be administered by injection comply with the requirements for *Labeling* ⟨7⟩, *Labels and Labeling for Injectable Products*. In addition, the label on the final container for each biologic states the following: the title or proper name (the name under which the product is licensed under the Public Health Service Act); the name, address, and license number of the manufacturer; the lot number; the expiration date; and the recommended individual dose for multiple-dose containers. The package label includes all of the above, with the addition of the following: the preservative used and its amount; the number of containers, if more than one; the amount of product in the container; the recommended storage temperature; a statement, if necessary, that freezing is to be avoided; and such other information as the Food and Drug regulations may require.

Packaging and Storage—The labeling gives the recommended storage temperature (see *General Notices*). Precautions should be taken where products labeled to be stored at a temperature between 2° and 8° are stored in a refrigerator, in order to assure that they will not be frozen. Diluents packaged with biologics should not be frozen. Some products (as defined in Section 600.15) are to be maintained during shipment at specified temperatures.

Expiration Date—For compendial articles the expiration date identifies the time during which the article may be expected to meet the requirements of the Pharmacopeial monograph, provided it is kept under the prescribed storage conditions. This

date limits the time during which the product may be dispensed or used (see *General Notices*, page 1). However, for biological products, the stated date on each lot determines the dating period, which begins on the date of manufacture (Section 610.50) and beyond which the product cannot be expected beyond reasonable doubt to yield its specific results and to retain the required safety, purity, and potency (Section 300.3 (1) and (m)). Such a dating period may comprise an in-house storage period during which it is permitted to be held under prescribed conditions in the manufacturer's storage, followed by a period after issue therefrom. The individual monographs usually indicate both the latter period and (in parentheses) the permissible in-house storage period. If the product is held in the manufacturer's storage for a longer period than that indicated (in parentheses), the expiration date is set so as to reduce the dating period after issue from the manufacturer's storage by a corresponding amount.

⟨1043⟩ ANCILLARY MATERIALS FOR CELL, GENE, AND TISSUE-ENGINEERED PRODUCTS

INTRODUCTION

A wide variety of reagents and materials, many of which are unique or complex, are required for the manufacture of cell, gene, and tissue-engineered products. These materials include plasma- or serum-derived products, biological extracts, antibiotics, cytokines, culture media, antibodies, polymeric matrices, separation devices, density gradient media, toxins, conditioned media supplied by "feeder cell layers," fine chemicals, enzymes, and processing buffers. Many of these items are used to ensure the survival and promote the growth of certain cell populations, although their mechanism of action may not be entirely understood. Examples include fetal bovine serum (FBS) and various media supplements. Other items, such as highly purified cholera toxin, are introduced into the processing stream during manufacturing to exert a specific biochemical effect and are immediately washed out in subsequent processing steps to avoid unwanted toxicity at a later point. The finished biological products produced in such processes are often complex mixtures that, in some cases, cannot be completely characterized. Careful scrutiny of the materials used in manufacturing is necessary to prevent the introduction of adventitious agents or toxic impurities, as well as to ensure the ultimate safety, effectiveness, and consistency of the final product.

In cell, gene, and tissue-engineered product manufacturing, these reagents and materials are collectively called ancillary materials (AMs). AMs have also been referred to as ancillary products, ancillary reagents, processing aids, and process reagents. AMs were first discussed under the synonym ancillary products in the U.S. Food and Drug Administration Notice, "Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products" (Federal Register 58(197), October 14, 1993, pp. 53248–53251). This document established the FDA's authority to regulate human somatic cell therapy products and gene therapy products. AMs are also synonymous with "processing materials" that were defined in 21 CFR Part 1271, "Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule" (Federal Register 66(5), January 8, 2001, pp. 1508–1559). AMs can be analogous to "components," and in some cases, "containers" as described in the current good manufacturing practice (cGMP) regulations for finished pharmaceuticals as outlined in s21 CFR 211.80 through 211.94 and 211.101(b) and (c).

The defining property of AMs is that they are not intended to be present in the final product. They are materials used as processing and purification aids or agents that exert their effect on the therapeutic substance. Materials or components that are intended to be in the final product dosage form (e.g., genetic materials, biopolymeric supports, physiological buffers) are not AMs. Cell banks and virus banks are also not considered AMs; there are a number of guidances that describe requirements for their certification. However "helper" viruses and "helper" plasmids may be considered AMs when they are not intended to be part of the final product.

The quality of an AM can affect the stability, safety, potency, and purity of a cell, gene, or tissue-engineered product. For example, the mechanism by which an AM exerts its effect may not be known, and the impact of normal variation of the AM on the quality and safety of the therapeutic product may not be understood. Alternatively, AMs of human or animal origin may present an infectious disease transmission risk. Other AMs, if administered to humans, may cause an immune reaction. Finally, an AM with toxic properties that is introduced into a manufacturing process and is not adequately removed in subsequent processing steps will expose the patient to a toxic substance and may impair the effectiveness of the therapeutic entity. These risks to the quality and safety of the therapeutic product are often heightened with cell, gene, and tissue-engineered products, due to the limited ability to conduct extensive in-process and release tests. For example, lack of in-process holding steps or limited shelf life may create the need to administer the cell, gene, or tissue-engineered products before in-process or final-release testing results are available. In other cases, the scarcity of suitable donor tissue or the complex logistics in the transport of biological materials may limit the amount of material available for testing. To minimize these risks, whenever possible, it is necessary to implement rigorous material qualification and prudent application of manufacturing process controls.

Frequently, these novel therapeutic products are created using complicated biological processes. The AMs employed in these procedures may be selected primarily for their unique functional contributions or biological effects. Whenever possible, it is preferable to source AMs that are approved or licensed therapeutic products because they are well characterized, have an

established toxicological profile, and are manufactured according to controlled and documented procedures. Conversely, the AM may be intended "for research use" and may, therefore, lack the level of qualification necessary for use in the production of a therapeutic product. In either case, the manufacturer of the cell, gene, or tissue-engineered product should develop comprehensive and scientifically sound qualification plans to ensure the traceability, consistency, suitability, purity, and safety of the AM. In cases where AMs are products approved for use for therapeutic purposes, the level of qualification will probably be less extensive than that for a material intended for research purposes. However, their suitability in the manufacturing process will still need to be established when the AM is being used beyond the scope of its intended use or labeling. The purpose of this chapter is to provide guidance in developing appropriate qualification programs for AMs employed in cell, gene, and tissue-engineered product manufacturing.

QUALIFICATION OF ANCILLARY MATERIALS

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific AM. The responsibility for AM qualification resides with the developer or manufacturer of the cell, gene, or tissue-engineered product. This section outlines the basis by which a manufacturer can establish rational and scientifically sound programs for qualifying AMs, although the broad nature of the cell, gene, and tissue-engineered products and of the AM used to produce these products make it difficult to recommend specific tests or protocols for a qualification program. Thorough documentation is the cornerstone of any qualification program.

A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety is the primary focus. In the later stages, AM production and qualification activities should be comprehensively developed to support eventual licensure of the cell, gene, and tissue-engineered product. On some occasions, complex or unique substances that have been shown to be essential for process control or production may not be available from suppliers that produce them in compliance with cGMP. In these situations, the manufacturer will have to develop a scientifically sound strategy for qualification. A qualification program for AMs used in cell, gene, and tissue-engineered product manufacturing should address each of the following areas: (1) identification, (2) selection and suitability for use in manufacturing, (3) characterization, (4) vendor qualification, and (5) quality assurance and control.

Identification

The first step in any qualification program is the listing of all of the AMs used in a given product manufacturing and where in the manufacturing process they are to be employed. The source and intended use for each material should be established, and the necessary quantity or concentration of each material should be determined. Also, alternate sources for each material should be identified.

Selection and Suitability for Use

Developers of cell, gene, and tissue-engineered products should establish and document selection criteria for AMs and qualification criteria for each vendor early in the design phase of product development. Selection criteria should include assessments of microbiological and chemical purity, identity, and biological activity pertinent to the specific manufacturing process. It is important to address these issues early in product development because certain AMs that are initially considered necessary may be impossible or prohibitively expensive to qualify, thereby justifying the investigation of alternatives or replacements. Examples include some animal- or human-derived materials that in some cases have alternate (i.e., plant or chemically synthesized) sources.

AMs of animal or human origin should be selected cautiously due to the potential infectious or zoonotic disease risks associated with these materials. Vendors should be selected that can supply documentation regarding the country of origin for animal-derived AMs to address concerns regarding transmissible spongiform encephalopathies and other diseases of agricultural concern, like tuberculosis and brucellosis. In many cases, the chain of custody for animal-derived AMs (i.e., abattoir → intermediate processing center → final processing center) will need to be documented. Vendors of human-derived AMs should be able to supply documentation regarding material traceability. For instance, human plasma-derived AMs should be sourced from licensed facilities that control the donor pool and appropriately screen the individual donors for relevant human infectious diseases. In some cases, vendors of animal- and human-derived AMs supply different grades of materials, some of which will be more suitable for use in cell, gene, and tissue-engineered product manufacturing than other grades. For example, FBS can be obtained that has been processed to reduce the risk of bovine viral contamination by subjecting it to validated irradiation and nanofiltration processes. Also, many animal and human plasma-derived components are subjected to chemical (detergent or solvent treatment) or physical (heat exposure for extended periods of time) treatments that have been shown through validation studies to significantly reduce the risk of adventitious microbial or viral contamination associated with starting AMs. Such AMs are preferred for use in cell, gene, and tissue-engineered product manufacturing processes because they significantly reduce the risks associated with the original material.

The complexity of risk assessment can be reduced by employing one of a number of quantitative or semiquantitative approaches, such as failure mode effects analysis (FMEA), quality function deployment (QFD), or hazard analysis and critical con-

General Chapters

trol point (HACCP). These programs typically assign a point value to each risk parameter for an AM that results in cumulative scores that make it easier to prioritize effort and resources for decreasing the risks associated with AMs. For example, an AM that has a strong safety profile and is used in minimal amounts in upstream steps of the manufacturing process and is thoroughly washed from the system would accumulate a low point score. Conversely, an AM that is known to be toxic and is employed in downstream processing would, therefore, possess a higher potential for appearing as a residual in the final product and would be assigned a higher point value. One can also assign points based on the risk classification (see *Risk Classification*).

Characterization

Specific quality control characterization tests need to be developed or adopted and implemented for each AM. The set of tests for each AM should assess a variety of quality attributes, including identity, purity, functionality, and freedom from microbial or viral contamination. The appropriate level of testing for each AM is derived from its risk assessment profile and the knowledge gained during development. Test specifications should be developed for each AM to ensure consistency and performance of the manufacturing process. Acceptance criteria should be established and justified on the basis of the data obtained from lots used in preclinical and early clinical studies, lots used for demonstration of manufacturing consistency, and relevant development data, such as those arising from analytical procedure development and stability studies.

Some AMs that are biological in nature may be difficult to fully characterize. Because these materials exert their effects through complex biological activities, and biochemical testing may not be predictive of the AM's process performance, functional or performance testing may be needed. Performance variability of such materials may have a detrimental impact on the potency and consistency of the final therapeutic product. Examples of complex functionality testing for AMs include growth promotion testing of individual lots of FBS on the cell line used in manufacturing, performance testing of digestive enzyme preparations, and in vitro tissue culture cytotoxicity assays. (see aspects of *Performance Testing*).

Vendor Qualification

Vendors supplying AMs should be qualified at the earliest opportunity. An early audit of the vendor's manufacturing facility, including their GMP and AM testing program, are basic elements of a vendor qualification program. A review of the vendor's processing procedures and documentation program is essential in establishing confidence in the vendor as a reliable supplier. Additionally, vendors that have been certified through an ISO inspection program or audited by other governmental agencies tend to have robust quality systems in place. Reports of past audits of U.S. suppliers obtained through the Freedom of Information (FOI) Act may augment the qualification process.

It is important to develop a good working relationship with a vendor. In some cases, the vendor may provide higher manufacturing standards, custom formulation services, or replacement of substandard components upon request, with or without additional costs. A good rapport is essential if further investigation into AM suppliers is warranted. It is also critical to ensure that the vendor takes appropriate steps to prevent cross contamination between its products during manufacture. Vendors should be familiar with the principles of validation, especially cleaning validation, as well as viral inactivation and sterilization validation. Finally, systems should be established where vendors supply written certification of processing or sourcing changes to customers, well in advance of the implementation of the changes so that customers can evaluate the potential impact of such changes.

Quality Control and Quality Assurance

Because the components of the qualification program are multifaceted and need to be in compliance with cGMP, they should be monitored by a quality assurance/quality control unit (QAU). Typical QAU activities include the following systems or programs: (1) incoming receipt, segregation, inspection, and release of materials prior to use in manufacturing, (2) vendor auditing and certification, (3) certificate of analysis verification testing, (4) formal procedures and policies for out-of-specification materials, (5) stability testing, and (6) archival sample storage.

RISK CLASSIFICATION

A scientifically sound and rational qualification program should be designed for each AM and should take into account the source and processes employed in its manufacture. Whenever available, AMs that are approved or licensed therapeutic products are preferable because they are well-characterized with an established toxicological profile and are manufactured according to controlled and documented procedures. Licensed biologics, approved drugs, and approved or cleared medical devices or implantable materials that have been incorporated into cell, gene, or tissue-engineered product manufacturing processes present a known or more favorable safety profile for the patient than nonapproved or nonlicensed versions. Qualification programs for these AMs should reflect the extensive scrutiny that these items were subjected to in their development and manufacture. Consequently, greater emphasis should be placed on the investigation of the impact of inherent variability of these AMs on final product function. For instance, a manufacturer may utilize human serum albumin, intended for human administration, as a supplement to a cell cultivation medium for a cell-based product. Because the cell-based product is marketed as a

licensed biological, one need not repeat all the testing already performed by the supplier as part of material qualification. In contrast, the impact of lot-to-lot variability on cell growth rate or maintenance of an important differentiated cellular property may be a prudent area of investigation. Alternatively, the stability of this material at the concentration employed in processing or its potential for interaction with other processing components may also be areas worthy of investigation. Such approaches to AM qualification therefore focus on the AM as a potential source of variability that may influence final product potency and safety. Qualification programs for these AMs should be comprehensive to minimize consumer risk and ensure that unacceptable lots or adulteration will be detected.

The qualification program must also take into account the quantity of the AM employed in manufacturing as well as its point of introduction in the manufacturing process. A relevant example is the use of FBS as a supplement to a tissue culture medium used to expand a stem cell population from a specific tissue for eventual administration to a patient (see *Manufacturing Overview* under *Cellular and Tissue-Based Products* ⟨1046⟩). A qualification program for such an AM would include (a) assurance that the serum was sourced from a country or region known to be free of bovine spongiform encephalopathy (BSE); (b) assurance that the source herds are monitored and test negative for specific diseases relevant in agricultural settings (e.g., tuberculosis, brucellosis, foot and mouth disease); (c) testing of the serum for sterility, mycoplasma, endotoxin content, and adventitious bovine viruses known to be associated with the material;[1] (d) the review and archiving of the supplier's certificate of analysis; (e) lot-to-lot assessment of the ability of the serum to consistently expand a representative cell population using a standardized cell culture quality control assay; and (f) on-site audit of the supplier to ensure that the material is sourced and processed in a manner deemed acceptable by a responsible QA unit.

To aid manufacturers and developers in the design of their qualification programs for a variety of AMs, tiers of sample risk categories are presented in *Tables 1–4* and are provided as a guide. Risk is also dependent on the amount and the stage at which the AM is used in the manufacturing process. *Tables 1–4* do not address the impact of quantity or stage of use.

General Chapters

Tier 1

These AMs are low-risk, highly qualified materials that are well-suited for use in manufacturing. The AM is either a licensed biologic, an approved drug, an approved or cleared medical device, or it is intended for use as an implantable biomaterial. Generally these components or materials are obtained as a sterile packaging system or dosage form intended for their label use, but are instead utilized "off label" in the manufacturing process for the cell, gene, or tissue-engineered product.

Tier 2

These AMs are low-risk, well-characterized material that are well-suited for use in manufacturing. Their intended use is for drug, biologic, or medical device manufacture, including cell, gene, and tissue-engineered products as AMs, and they are produced under relevant cGMPs. Most animal-derived materials are excluded from this category.

Tier 3

These AMs are a moderate risk material that will require a higher level of qualification than previous tier materials. Frequently, these materials are produced for in vitro diagnostic use and are not intended for use in the production of cell, gene, or tissue-engineered products. In some cases, upgrade of AM manufacturing processes may be necessary in order to employ the AM in manufacturing of these products (e.g., modification of the production process for a diagnostic grade monoclonal antibody to include robust viral removal steps in purification).

Tier 4

This is the highest risk level for AMs. Extensive qualification is necessary prior to use in manufacturing. The material is not produced in compliance with cGMPs. AMs are not intended for use in the production of cell, gene, or tissue-engineered products. This risk level includes highly toxic substances with known biological mechanisms of action, and also includes most complex, animal-derived fluid materials not subjected to adventitious viral removal or inactivation procedures. These materials may require (a) an upgrade of AM manufacturing processes; (b) treatment of AMs to inactivate or remove adventitious agents, disease-causing substances, or specific contaminants (e.g., animal viruses, prions); (c) testing of each lot of material to ensure that it is free of adventitious agents, disease-causing substances, or specific contaminants; (d) validation of the manufacturing process of the cell, gene, or tissue-engineered product to assess consistency of removal of a known toxic substance or lot-release testing to demonstrate reduction levels considered to be safe; or (e) validation of the manufacturing process of the cell, gene, or tissue-engineered product to assess consistency of removal or inactivation of adventitious agents, disease-causing substances, or specific contaminants associated with the material. Developers in the early stages of development should evaluate the necessity of these materials and explore alternative substances or sources.

[1] Most suppliers test for adventitious agents according to 9 CFR 113, which was developed by the Center for Veterinary Biologics, Animal and Plant Health Inspection Service, United States Department of Agriculture. These tests may differ from those used to test products developed for human use (e.g., mycoplasma).

Table 1. AM Risk Tier 1
Low-Risk, Highly Qualified Materials with Intended Use as Therapeutic Drug or Biologic, Medical Device, or Implantable Material

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant insulin for injection	Cell culture medium additive	DMF cross reference (when possible or practical)
Organ preservation fluid	Process biological fluid employed in tissue transport or processing	Certificate of analysis
Human serum albumin for injection	Cell culture medium	Assess lot-to-lot effect on process performance[1]
Sterile fluids for injection	Process biological fluid employed in tissue transport, cell processing, purification	Assess removal from final product
Implantable biomaterials (formed collagen, silicone, polyurethane constructs intended for surgical implantation)	Scaffolds, matrices for immobilized cellular cultivation	Stability assessment on AM as stored for use in manufacturing[2]
Recombinant deoxyribonuclease for inhalation or injection	Process enzyme employed in viral vector manufacturing, stem cell processing	
Antibiotics for injection[3]	Cell culture medium and biopsy transport fluid additive to reduce risk of bacterial contamination	
Injectable monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Injectable cytokines	Cell culture medium	
Vitamins for injection; defined nutrients, chemicals, or excipients intended for injection	Cell culture medium additive employed in cell expansion, controlled cellular differentiation/activation step, or manufacture of a viral vector	
IV bags, transfer sets and tubing, cryopreservation bags, syringes, needles	Storage vessels or container closure systems, closed aseptic transfer systems	

[1] See *Performance Testing.*
[2] Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrate the stability and preservation of activity of the AM under the conditions that are specific to the manufacturing application.
[3] Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity.

Table 2. AM Risk Tier 2
Low-Risk, Well Characterized Materials with Intended Use as AMs, Produced in Compliance with GMPs

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant growth factors, cytokines[1]	Cell culture medium additive	DMF cross reference (when possible or practical)
Immunomagnetic beads	Immunomagnetic separation of cells	Certificate of analysis
Human AB serum	Cell culture medium additive	Assess lot-to-lot effect on process performance[2]
Progesterone, estrogen, vitamins, purified chemicals (USP-grade)	Cell culture medium additives, induction agents, buffer components	Assess removal from final product
Sterile process buffers	Process biological fluid employed in tissue transport, cell processing, purification	Stability assessment on AM as stored for use in manufacturing[3]
Biocompatible polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	When relevant, confirm certificate of analysis test results critical to product (could include functional assay)
Proteolytic enzymes	Process enzyme	Vendor audit
Tissue culture media	Cell culture medium additive	
Monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Density gradient media	Cell separation via centrifugation	

[1] These AMs should be produced from nonmammalian, recombinant sources (i.e., microbially grown in the absence of animal-derived growth medium components).
[2] See *Performance Testing.*
[3] Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrates the stability and preservation or activity of the AMs under the conditions that are specific to the manufacturing application.

Table 3. AM Risk Tier 3
Moderate-Risk Materials Not Intended for Use as AMs
(frequently produced for in vitro diagnostic use or reagent grade materials)

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant growth factors, cytokines	Cell culture medium additive	DMF cross reference (when possible or practical) Certificate of analysis Assess lot-to-lot effect on process performance[1] Assess removal from final product Stability assessment on AM as stored for use in manufacturing[2] When relevant, confirm certificate of analysis test results critical to product (could include functional assay)
Tissue culture media	Cell culture medium additive	Vendor audit
Monoclonal antibodies (diagnostic-grade produced in cell culture)	Immunologically targeting specific cell populations for selection or removal	Upgrade manufacturing process for material to GMP
Process buffers	Process biological fluid employed in tissue transport, cell processing, purification	Develop stringent internal specifications
Novel polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	Determine if lot-to-lot biocompatibility, cytotoxicity, or adventitious agent testing are needed
Proteolytic enzymes	Process enzyme	
Purified chemicals (reagent-grade)	Culture medium additives, induction agents, buffer components	

[1] See *Performance Testing*.
[2] Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrate the stability and preservation or activity of the AM under the conditions that are specific to the manufacturing application.

Table 4. AM Risk Tier 4
High-Risk Materials

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product	Qualification or Risk Reduction Activities
FBS	Cell culture medium additive	Same as in *Table 3*, plus
Animal-derived (including human) extracts	Cell culture medium additive	
Animal-derived polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	Verify traceability to country of origin
Purified enzymes	Process enzyme	
Ascites-derived antibodies or proteins	Immunologically targeting specific cell populations for selection or removal	Assure country of origin is qualified as safe with respect to source-relevant animal diseases, including TSE
Animal or human cells used as feeder layers	Cell culture substratum or source of medium components	
Chemical entities with known toxicities (i.e. methotrexate, cholera toxin, *Staphylococcus aureus* pore-forming hemolysin, *Staphylococcus* enterotoxins A and B, toxic shock syndrome toxin)	Selection agents used in cell culture to improve or maintain transgene expression, enhance cellular proliferation, improve cell survival upon cryopreservation, superantigens for the activation of T cells	Adventitious agent testing for animal source-relevant viruses

PERFORMANCE TESTING

In cases where AMs are chosen for their ability to provide a particular biological function in producing the therapeutic product, performance testing becomes an essential component of their overall qualification. This is especially true when the AM plays a critical role in modulating a complex biochemical effect and has a large impact on product manufacturing yield, purity, or final product potency. These AMs tend to be complex substances or mixtures, are frequently biologically sourced, and can exhibit significant lot-to-lot variability. As a result, these AMs usually have no simple identity test, nor can they be easily characterized by physical or chemical tests. The development of well-defined performance assays for complex AMs will not only ensure process reproducibility and final product quality, but in many cases will satisfy the identity testing criteria in accordance with 21 CFR 211.84(d).

In some cases, the initial qualification of an AM for use in manufacturing should be the investigation of the effect of the amount of the AM on the desired response (increased yield, purity, or potency of the therapeutic product). The amount of the AM used in manufacturing should be chosen to consistently yield the desired effect while minimizing issues by removing the AM in subsequent processing steps. Such testing frequently assesses the important functional attribute expected of the AM in a scaled-down or simulated manufacturing process. Some examples follow:

- If an AM is added to the culture media because it promotes cellular proliferation or the secretion of a critical therapeutic agent, the assay could demonstrate that each lot of AMs produces the expected rate and amount of cellular proliferation or the expected level of secreted therapeutic agent.
- If a monoclonal antibody is used to purify a particular cell type, the new lot of monoclonal antibody could be shown to purify the cell population with the expected recovery and purity for the desired cell type.
- If a deoxyribonuclease is used to degrade cellular DNA, new lots could be tested for the ability of the deoxyribonuclease to degrade DNA.
- If a particular type of density gradient material is used to purify a vector or cell, new lots of the material used to make the gradient could be shown to purify the vector or cell to an acceptable level.
- If a plasmid or viral vector is used in the production of a gene therapy vector (e.g., helper function), new lots of the helper vector could be shown to produce the expected amounts of the gene therapy vector.
- If a cell therapy is produced in a hollow-fiber bioreactor, new lots of the bioreactor could be shown to produce the anticipated amount of cell product.

The actual assay used may well evolve as the manufacturing process is developed further and the critical relationships of the AM and the final product are better understood.

Because most performance testing yields relative results, it is often helpful to assay a new lot of AMs side by side with an approved lot of AMs or an official reference standard, if available. This simultaneous comparison helps to reduce the variability due to different lots of cells or vectors and will help discern variability associated with the different lots of AMs. If performance testing involves assays to demonstrate that the new lot of AMs does not affect the impurity profile of the final therapeutic product, either by generating new impurities or by increasing the level of existing impurities, it is helpful to assay both for the total level of impurities, as well as look for the presence of new impurities. An immunologically-based binding assay can typically assess only the total level of impurities. For example, a Western blot of the gene therapy product that is probed both with antibodies to the product and antibodies to host cell proteins is useful for detecting new protein species and significant increases in the levels of host cell impurities. This initial qualification is enhanced by a performance assay that has a quantitative readout with a clear change in the signal when a significant change in the amount of AMs is introduced into the assay (e.g., dose response). A threshold-type response (i.e., there are two levels of response to the AM and neither large changes in an AM below a certain dose nor above a certain dose change the response) can make it more difficult to select a concentration of AM that consistently results in the desired effect and minimizes the residual levels of the AM in the final therapeutic product.

ANCILLARY MATERIALS RESIDUAL LEVEL ASSESSMENT AND REMOVAL

AMs are not intended to be present in the final dosage form in cell, gene, and tissue-engineered products. Their presence in the final product could lead to undesired effects in the recipient or have a detrimental effect on product potency. Undesired effects in humans include direct toxicity of the AM or an unwanted immunogenic response. Some examples include the following:

- In the generation of a tumor vaccine using a patient's tumor biopsy as the starting material, a chemical entity is introduced to denature the cell surface proteins and tumor antigens to enhance their antigenicity. The chemical entity is known to be highly toxic.
- Antibiotics may be added to a transport solution for human cells to address microbial contamination issues associated with the procurement procedure. Residual levels of the antibiotic may affect the proliferative capacity of the final engineered cellular product. Residual antibiotics could also cause an anaphylactic response in some individuals.
- FBS, employed in the cultivation of an engineered human skin graft, may cause the development of a humoral antibody response directed against bovine proteins.
- Aggregated mouse immunoglobulin, a trace impurity in a purified preparation of mouse monoclonal antibody used to target a cell population for immunoselection, may be immunogenic.
- A cytokine, employed as an immunomodulator in the generation of a gene-modified autologous tumor vaccine product, may elicit a severe reaction in the recipient.
- Cholera toxin, employed as part of a cell culture medium for a cell therapy product intended for intravenous administration, will be highly toxic to the recipient if it is not removed during processing.

These risks can be mitigated through the design of processes to include steps to adequately remove the AM through dilution, separation, or inactivation, as well as the development of analytical detection assays to assess the AM levels during processing and in the final therapeutic product. Assessment and removal strategies for residual AMs should be considered in the early phases of process development. There are two different approaches for assessing residual AM levels in the final therapeutic product: (1) Validation studies can demonstrate that the process is capable of removing more of the AM than would be present in a worst-case scenario. (2) The residual levels of an AM can be measured for each lot at an appropriate step in the manufacturing process.

General Chapters

Validation of an AM removal is often best performed by spiking the impure product with "worst case" or higher levels of the AM and showing the purification process is capable of removing the AM to "undetectable levels." Clearance factors can then be generated for each purification step in a manner analogous to that done in viral clearance studies. When designing the validation studies, the following three considerations should be included: (1) The assay should be able to accurately quantitate the AM in each sample matrix. (2) If the validation is conducted at a scale smaller than that used for routine lot production, the comparability of this smaller scale process to the full scale process needs to be demonstrated. This usually means that the smaller scale process is operated using the same critical parameters as the full scale process with the product generated at each step having a similar purity and yield. (3) As with any spiking study, one has to demonstrate that the additional, higher level of AM has not affected the purification process. If the second approach of measuring residual levels of the AM in each lot is used, the specification for the maximum amount of AM in the final therapeutic product is based on the amount of the AM in the lots used in toxicological or clinical studies or known toxicological data.

The development of sensitive and reproducible analytical assays for AMs is another important component of a risk reduction approach. Two types of assays are useful in assessing the levels of residual AM impurity: a limit test and a quantitative test. Either test should be accurate, precise, robust, and have a low limit of detection. Assays for residual AMs may be performed on the product before it is formulated (e.g., on the drug substance) to avoid any interference of the components used in the formulation with the assay for residual AMs or in the final drug product. Spike-recovery controls are often included in such assays to demonstrate that the sample matrix does not inhibit the detection of the AM. Preferably, assays should be designed to detect all forms of AMs including aggregates, fragments, or conjugates. Aggregated protein has been shown to be particularly immunogenic.

Immunoassays such as ELISA are most commonly used to assess residual levels of AMs. An ELISA for bovine serum albumin (BSA) has been used to assess residual levels of FBS. Polymerase chain reaction (PCR) technology has been employed to assess residual levels of host cell DNA. Labeling cells with ^3H thymidine or performing PCR for a feeder cell-specific gene sequence are two ways to assess for residual levels of feeder cells. If "wash out" of the AM is achieved by exhaustive dilution associated with further processing activities, it may be useful to calculate the dilution factor for the AM during this processing. In some cases, this is sufficient to ensure that the AM has been reduced to safe levels for early clinical development. Data should be obtained later in clinical development to confirm the wash out of the AM at the expected step(s). This approach is particularly useful when there is pre-existing knowledge of the therapeutic levels and toxicity of the AM. In other cases, information regarding the safety and tolerability of the AM should be collected (in preclinical toxicology studies or later with human clinical studies) in order to determine the safe or nontoxic levels that must be achieved. These data may be needed even for an AM that is approved for use for therapeutic purposes if it is being used in a manner inconsistent with its intended use or labeling or if the route of administration or dosage level of the AM may present risks not previously encountered or considered.

CONCLUSION

While many types of AMs are used during the manufacture of cell, gene, and tissue-engineered products, they have received less emphasis than the final products. However, the importance of AM quality to the quality of the final product cannot be overstated. Good quality AMs should perform as intended in a consistent manner, batch-to-batch, if they are carefully selected and appropriately used. AMs of insufficient quality will affect the quality and the effectiveness of the final product and endanger the health of patients. Thus, implementing an AM qualification program that addresses the risks associated with the AM, the stage of manufacture at which it is used, and the amount of the AM used during manufacture will ensure the safety and effectiveness of the final product.

APPENDIX

Relevant USP Chapters and Regulatory References

AMs used in cell, gene, and tissue-engineered products will be regulated in the context of the manufacturing process of the cell, gene, and tissue-engineered products. Certain AMs may already be approved for uses other than for cell, gene, and tissue-engineered product manufacture. It is preferable to source AMs that are approved therapeutic products when they are available because they are well-characterized with an established toxicological profile and are manufactured according to controlled and documented procedures. The following list of documents should provide relevant regulatory guidance and a description of best practices in product and process development, manufacturing, quality control, and quality assurance:

- *Biological Reactivity Tests, In Vitro* ⟨87⟩
- *Biological Reactivity Tests, In Vivo* ⟨88⟩
- *Cellular and Tissue-Based Products* ⟨1046⟩
- *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩
- *Capillary Electrophoresis* ⟨1053⟩
- *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩
- *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩
- *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩
- *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩

- 21 CFR 211 Subpart E, 211.80 through 211.94 and 211.101
- 21 CFR 312
- 21 CFR 314
- 21 CFR 801.109 (b) (1)
- 21 CFR 807.81 through 21 CFR 807.97
- 21 CFR 812
- 21 CFR 814
- FDA Center for Biologics Evaluation (CBER) "Draft Guidance for Monoclonal Antibodies Used as Reagents in Drug Manufacturing" (1999)
- FDA Center for Biologics Evaluation (CBER) "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993)
- FDA Center for Devices and Radiological Health (CDRH) "Class II Special Controls Guidance Document: Tissue Culture Media for Human ex vivo Tissue and Cell Culture Processing Applications; Final Guidance for Industry and FDA Reviewers" (May 16, 2001)
- CDRH Blue Book Memorandum G95-1
- ISO 10993-1: 1997 "Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing"
- International Conference on Harmonization (ICH) Q5A "Guidance for Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human and Animal Origin"
- International Conference on Harmonization (ICH) Q5D "Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products"
- Public Health Service Guideline on Infectious Diseases Issues in Xenotransplantation (October 18, 2000)

⟨1044⟩ CRYOPRESERVATION OF CELLS

INTRODUCTION

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability. The purpose of cryopreservation is to bank the cells and allow their future use in in vitro or in vivo applications for which post-thaw function is sufficiently representative of the cells' prefreeze function. Cryopreservation also minimizes the risk of genetic mutation or development of subpopulations due to cell replication. Depending on the application, sufficient post-cryopreservation function may be assessed by the ability to divide, proliferate, differentiate, express genes, or to produce proteins, or by another specific functional property.

This chapter presents best practices for cryopreservation, maintenance, and use of a wide range of cells, cell therapy products, and cell banks derived from a variety of sources including human, animal, and microbial cultures (the chapter also contains an *Appendix* with additional guidance documents that are useful for particular cell types and applications). Cryopreserved cells provide a ready source of viable cells that can be used, either directly or indirectly, for the purposes of diagnostic tests, therapy, manufacture of drug products and vaccines, and for bioassays used to evaluate the potency of therapeutic drugs and vaccines. In some cases the cells themselves, after cryopreservation and thaw, constitute the patient therapy, and in other cases the cells are propagated or otherwise manipulated ex vivo in order to generate the product (e.g., a culture-expanded cellular therapy, a therapeutic protein, a monoclonal antibody, or a vaccine). In all cases, proper cryopreservation is essential for retention of required cellular properties and, ultimately, for application toward the advancement of patient therapies.

PRINCIPLES OF CRYOPRESERVATION

Overview

Understanding the role of water and the need to adequately remove it from cells or abrogate its ability to form ice crystals, which damage the cell membrane, is critical to successful cryopreservation. When cells are frozen in aqueous suspension, often they are destroyed. However, in the 1940s Polge and others discovered the cryoprotective properties of glycerol. Since then several chemicals, generically called *cryoprotectant agents* (CPAs), have been identified. The mechanism of action of CPAs is complex and is not fully understood. However, according to the commonly accepted theory of *colligative* action, CPAs increase solute concentration both within the cell and extracellularly, thereby suppressing ice formation. For this purpose, the so-called penetrating (or intracellular) CPAs [e.g., dimethylsulfoxide (DMSO), glycerol, propanediol, and methanol] must be able to cross the cell membrane readily and penetrate the cell without significant toxicity. There also is a group of nonpenetrating (or extracellular) CPAs (e.g., sucrose and trehalose) whose mechanism of action is thought to be related at least in part to their stabilizing interaction with cell membranes. This property also may explain the cryoprotective activities of certain large molec-

ular weight compounds such as hydroxyethyl starch and polyvinylpropylene. Theoretical models of cryoprotection typically evoke the colligative theory, but full explanation of CPA action is yet to be established.

An alternative form of cell preservation, commonly called *vitrification,* whereby the cell suspension is loaded with high levels of penetrating CPAs (often several in combination), induces a glass-like state in which cellular and extracellular water cannot readily form ice crystals. When cell suspensions prepared in this way then are cooled very rapidly (cooling rates of 100°–1000°/min or more) the extreme viscosity prevents osmosis, and the water molecules are unable to form ice. This procedure has been widely used for complex structures including a variety of human, plant, and animal tissues and may help preserve those cell preparations that have variable degrees of cellular permeability or when standard cryoprotection cannot deliver the range of conditions required to optimally preserve viability in all the tissues' component cell types.

CPAs have biological activities beyond their cryoprotective properties. Some, like DMSO, can affect the cell membrane, cytoskeleton, and gene expression and may be toxic to cells following prolonged exposure. Therefore, during development of new cryopreservation protocols analysts should perform a toxicity assay in which the cells are exposed to the CPA over a range of time intervals to evaluate loss of viability or alteration of functionality.

Key Elements of Cryopreservation Practice

For any cellular sample or therapeutic product being cryopreserved, method development should address the following elements:

PREFREEZE PROCESSING AND CHARACTERIZATION

Optimizing the condition of the cells immediately before cryopreservation is critical to a successful outcome. The nature and extent of prefreeze processing depends on the state of the original cells harvested for preservation, the composition of the cell suspension, and the specific processing steps leading into cryopreservation. Prefreeze processing may include selection of subpopulations, ex vivo expansion, or incubation with activating or priming factors.

Precryopreservation characteristics and identity should be established during early process development. For cell banks in particular, the cell status and optimal growth conditions, as well as documented history (with traceability to a qualified cell bank or acceptable source), characteristics, and authenticity should be documented. Cell status and history typically are described in terms of the nature and number of manipulations and culture passages from the primary cells or original isolate. Finite or primary cells usually are cryopreserved at an early passage to maintain integrity of the original tissue, but continuous cell lines may be cloned and expanded, ensuring a homogeneous cell population. It is recommended to prepare cell banks from a single preparation or expanded population of cells since it is often necessary to pool cells for freezing from multiple culture vessels. Cells from cultures with different passage histories and certainly from different donors should not be pooled. In both cases, analysts should maintain detailed records of the procedures.

To prepare for cryopreservation of cultured cells, cells should be harvested during exponential or the most rapid phase of growth and before the culture enters stationary phase. Harvesting cells during this phase ensures that the cells are most viable and uniform. The optimal concentration of cells will depend on the cell type, purpose, and best recovery. Typically this lies between 10^6 and 10^7/mL for manufacturing cell banks but may be different for other purposes. Complete growth medium renewal a day before cell harvest also can be beneficial. Additionally, most cell suspensions benefit from washing by centrifugation and resuspension in an isotonic medium to a specific cell concentration. Prefreeze processing should not result in cells that are stressed before the start of the freezing process, or cell losses during freezing or after thaw will be higher than expected.

Optimizing the growth conditions of a cell line or primary cells is important to maintain high viability of the cells in culture. Typically, cells growing actively and in exponential phase have a low cytoplasm to nuclear volume ratio, which is conducive to successful cryopreservation with penetrating cryoprotectants. Suboptimal or improper culture conditions may result in lower viability and cell states that will be less robust for preservation and recovery. The culture medium should be optimized and the same medium should be used throughout all experiments, and each batch of animal-derived materials (e.g., serum) and other culture reagents should be qualified (e.g., see the 2010 WHO guidance and the FDA 2010 guidance referenced in the *Appendix*). If possible, it is recommended to not use animal-derived components in the culture medium particularly for cells used for therapy or as manufacturing substrates.

Per the WHO 2010 guidance and based on a risk assessment, either the Master Cell Bank (MCB) or the Working Cell Bank (WCB) must be tested for adventitious agents. Ideally, samples of cells should be tested for adventitious agents before freezing. The specific testing regimen for potential microbial or viral contamination of cells depends on the donor source, the culture history, and the intended use. Detailed records of the cell history should be maintained as a basis for appropriate risk assessment to direct supplementary testing that may be required (e.g., exposure to bovine viruses in bovine serum albumin). Specific regulatory requirements for testing of cells, or donors of the cells, for products intended for a particular use (e.g., cell therapy or vaccine manufacture) are based on past experience regarding key agents that must be included or considered. *USP* general chapter *Cellular and Tissue-Based Products* ⟨1046⟩ contains guidance about sterility and safety testing requirements for cell therapy products.

REAGENTS AND CONTAINERS

All cryoprotectants, containers, etc., should be fit for purpose as indicated in relevant regulatory guidances. Sterile, single-use, disposable plastic bags, cryovials, or straws are customarily used for cryopreservation. Manufacturers' specifications should be carefully reviewed to ensure that the material used to manufacture the cryocontainer is appropriate for use at the storage temperature, is chemically compatible with the contents, minimizes the potential for leachables and extractables, and assures container closure integrity. If straws are used, then primary or secondary containment during storage is important to prevent direct contact of the preserved cells with liquid nitrogen. Cryovials should be selected based on their ability to provide adequate cell bank integrity.

Preservation of cells typically requires the use of specialized solutions that contain a base (typically an isotonic saline-based solution) with CPAs (most commonly DMSO but sometimes glycerol) and sometimes proteins (fetal bovine serum, human serum or plasma, conditioned medium, or human albumin). The optimum composition for different cell types may need to be determined.

The types of vials, labels, ink, or markers used should withstand extreme liquid nitrogen temperatures. The markings on the label should be legible and barcoded if possible. The minimum information on the label should include name or description of cell population, date of cryopreservation, lot number, and passage number if needed. Since most cryolabels are very small, additional information can be included on associated documentation. In certain applications it may also be necessary to sequentially number vials within a single lot as part of the minimum information on the label, to enable better control over movement of vials from a single bank, and to identify sectors of the bank which may have received different cryopreservation conditions.

ADDITION OF CRYOPROTECTANT SOLUTION

Cryoprotectant solutions typically are hypertonic and are not physiological. For example, a 10% DMSO solution used commonly in cell preservation has a concentration of approximately 1.4 osmolarity (Osm/L). Cells introduced into this type of solution rapidly dehydrate as water leaves the cell in order to reduce the difference in osmotic potential between the inside and outside of the cell. DMSO slowly permeates the cell to re-equilibrate. This may cause excursions in volume that can result in a loss of cell viability. Therefore, cryopreservation solutions commonly are added to a cell suspension in stepwise additions or gradually (e.g., using a syringe pump) or slowly dispensing down the side of the container to prevent cell losses resulting from osmotic stress. The method for introducing or removing a cryopreservation solution should be developed and evaluated for its impact on cell viability and functionality.

In the case of DMSO, a large latent enthalpy of mixing results in sample heating when the two solutions are mixed. This heating can be high enough to damage the cells, so solutions that contain DMSO commonly are precooled before mixing. Prechilling the solution reduces heating associated with mixing of the solution, reduces the osmotic volume changes that the cells experience, and reduces cell losses associated with exposure to DMSO. The time that cells are exposed to the cryoprotectant, prior to freezing, should be limited and the maximum time allowable, without deleterious effects, should be determined during development work for routine use.

COOLING

Two different types of freezing typically are used for cells: controlled-rate cooling (using programmable freezers) and passive cooling (including use of insulated containers). Controlled-rate freezers are attached to liquid nitrogen supplies. The temperature of the chamber should be controlled by increasing or decreasing the flow of cold nitrogen gas into the chamber according to a preprogrammed step. Controlled-rate freezing protocols typically involve several steps, each of which should be evaluated and qualified for a specific cell type.

The use of controlled-rate freezing provides more precise control of the freezing environment and therefore may provide more consistent (and higher) post-thaw recovery for cells that may have a narrow range of cooling rates associated with maximum survival or cells that are sensitive to the temperature at which ice forms in the extracellular solution. Temperature probes placed near the cells being frozen, or in a mock cell suspension that undergoes cryopreservation simultaneously, are used to monitor the freezing process and to provide process control. If release of the latent heat of fusion is delayed or poorly controlled, cells undergoing cryopreservation may be damaged and may have diminished viability after thaw.

Disruption of the controlled-rate freezing during the protocol may occur and typically is caused by failure of a valve in the controlled-rate freezer or cryogen. Protocols for handling disruption of the freezing process and backup plans should be prepared.

Passive freezing involves placing a product in a freezer (about −80° or −150°) and permitting the sample to cool in an uncontrolled fashion. Insulation or specially designed boxes are used to slow the cooling rate for the sample. The average cooling rate achieved for the majority of the process and the consistency of freezing curves should be evaluated and qualified for purpose. In general, control of the thermal environment during freezing results in improved post-thaw recovery, but certain cells exhibit comparable post-thaw recovery when they are passively cooled.

CRYOGENIC STORAGE, SAFETY, AND TRANSPORT

After the freezing process has been completed, products are transferred from controlled-rate or mechanical freezers to cryogenic storage units. Some microbial cell cultures can be suitably maintained in mechanical freezers but this should be demonstrated. Sample warming should be minimized during transfer of the cell product from the freezing device to storage. Cold tables or insulated transfer devices can be used to minimize warming during transfer. Newly cryopreserved cells commonly are placed in a quarantine cryogenic storage unit before completion of testing for adventitious agents. After testing, cells that test negative for adventitious agents can be released for transfer into long-term cryogenic storage units.

The inventory system (or repository) for the maintenance of the cryopreserved cells should be designed for easy access to minimize specimen handling, and the number of times per day that a repository is accessed should be limited because exposure to warmer temperatures may compromise cell viability and, consequently, longer-term stability. Cell banks (e.g., MCBs) or other cell cultures that are accessed infrequently should be stored separately from WCBs or other cell cultures that are accessed more often. Frequent retrieval from the cell bank/culture may cause shifts in temperature. This activity must not compromise the long-term stability and performance of the infrequently used cell bank/culture. It is also valuable to divide a bank and store it in multiple locations to decrease risks due to a catastrophic event at a particular site.

When storing cryopreserved cells, analysts should ensure that the storage temperature does not rise above a critical temperature called the glass transition temperature. For long-term storage of fastidious specimens such as cell lines and primary cell cultures, this critical temperature is not warmer than −130° for nonclinical specimens or not warmer than −150° for clinical material (to give an adequate margin of error) in the vapor phase of the liquid-nitrogen freezer. Liquid-nitrogen freezers are prone to temperature gradients in the vapor phase based on the shape and design of the freezer and the level of liquid nitrogen. Although storing cryopreserved cells in liquid nitrogen prolongs longevity, hazards associated with unsuitable containers or container use (e.g., exploding vials and rupturing bags) have prompted greater use of nitrogen vapor phase storage. Liquid-nitrogen vapor phase provides a more convenient and safe environment for vial retrieval. If the liquid-nitrogen freezer is suitably configured, the working temperature in the vapor phase is commonly −150° or colder. The liquid-nitrogen freezer should be qualified, and the temperature of the vapor phase should be routinely checked to ensure that the temperature does not become warmer than −130° for cell lines or other frozen material or warmer than −150° for material used for clinical applications (e.g., cell therapies).

Temperature-monitoring systems should permit recording and storage of temperature history for quality control purposes. Storage units should be attached to alarms and facility monitoring systems. Critical storage units should be equipped with a multilevel alarm system to ensure backup in the monitoring and response. The storage units should be routinely monitored for temperature failure caused by power disruption and any other potential malfunctions. In the event of equipment or power failure, backup refrigeration should be available.

Proper operation of a repository requires monitoring of temperature and liquid-nitrogen levels and automatic filling. In addition, it is recommended to have a backup for emergency cooling (e.g., empty backup cryogenic storage) in case of freezer failure.

Only individuals who are trained for this purpose should access cryopreserved products or samples. In some cases, verification by a second person is required for source traceability. Personnel assigned to the implementation of the protocols should be trained in standard operating procedures (SOPs). Sample tracking systems that incorporate computer software and sometimes barcoding for identification, logging, and tracking of frozen samples are particularly useful for large sample repositories and may facilitate rapid retrieval of samples and minimize time that the entire repository is exposed to the risk of temperature excursions. In addition, it is recommended that all changes to cryostorage inventories be recorded in log books near the storage unit.

Products and samples such as primary cells, cell lines, and cell therapy products routinely are shipped among sites of collection, processing, storage, and use. Cryopreserved cells typically are shipped in liquid-nitrogen vapor shippers with temperature-monitoring systems to ensure that the unit does not become warmer than −130° for cell lines and −150° for clinical material during the shipping process. Shipping containers are subjected to significant vibrations and mechanical stresses during shipment and should be evaluated on a regular basis for proper function. Shipping validation studies should cover worst case scenarios and include temperature monitors for critical materials.

Cryopreserved cells, whether shipped nationally or internationally, should be transported using local postal, US Department of Transportation, and International Air Transport Association guidelines. Packages also should meet other regulatory requirements for quarantine, biosafety, and biosecurity. Cryopreserved cells should be retrieved, packed, and shipped in a manner that does not interfere with the integrity of the cells. For most cryopreserved cells, shipping in dry ice for short duration may be adequate, but the shipping process should be validated, shown to have no adverse impact on the cells, and temperature monitors should be included. However, some cells may require shipping in liquid-nitrogen vapor phase (Dewars). Prevalidation of the shipping methods may be required to determine the best option and prevalidation risk assessment should be performed even if only one option for transport is being considered. With the shipment, shippers should include instructions for proper storage upon cell receipt.

THAWING

Cells frozen using conventional methods (controlled rate or passive freezing) or by vitrification should be thawed as rapidly as possible, and the thawing process starts as soon as the frozen sample is removed from storage. Slow warming rates result in recrystallization damage or exposure of the cells to high extracellular concentrations of CPAs, either of which can result in cell death. For each cell therapy product and cell line the most appropriate thawing procedure (temperature, gradient, and time) needs to be developed. These products and cell lines typically are thawed in a warm-water bath or for therapeutic cell preparations in a bead bath or thermoblock. The water bath should be cleaned regularly and should contain sterile water or *Water for Injection*. The temperature of the bath also should be monitored. Many clinical labs use plastic overwrap bags to hold the primary container during rapid thaw to reduce the risk of product contamination in case the integrity of the inner container is impaired. Alternatively, warm bead baths (usually approximately 37°) can be used to reduce contamination risks. Thawing rates should be as rapid as possible (>1°/s for most mammalian cells). Increasing bath temperatures above 42° to increase the warming rate must be done with extreme caution because hyperthermic temperatures can damage cells, inducing necrosis or apoptosis.

POST-THAW PROCESSING AND EVALUATION

Because cryopreservation solutions are not physiological, it is not uncommon for some post-thaw processing to be performed. For cells preserved in DMSO, cells typically are washed or diluted immediately post-thaw because this CPA is harmful in particular to frozen and thawed cells. Cells are more sensitive to expansion than contraction, so CPA removal or dilution protocols must be carefully optimized to prevent cell losses from dilution or removal.

Quantifying the viability of cells post-thaw is important and may be performed by a variety of methods depending on the post-thaw requirements of the cells. Minimum viability limits should be set based on experience and thawed products with viabilities below the set limits should be discarded. The process of cryopreservation subjects the cells to significant stresses that can alter metabolic function, membrane structure, etc. Therefore, development and validation of suitable post-thaw assays are critical. Post-thaw function is most commonly assessed using physical integrity (e.g., membrane integrity), metabolic activity, mechanical activity (attachment or contraction), mitotic activity, or engraftment potential. The selection of assay depends strongly on the desired post-thaw function of the cell. Membrane integrity is used most often. Today, dyes like trypan blue are used less often to measure post-thaw physical integrity because the dye is difficult to validate on frozen and thawed cells. The method to test viability needs to be carefully selected and qualified for the particular cell type being measured with a protocol that specifies diluents and time. Fluorescent dyes are used with increasing frequency to determine cells' post-thaw physical integrity. Rigorous methods of post-thaw assessment typically involve multiple measures of cell viability and, in particular, at least two independent assays to measure post-thaw viability. For example, post-thaw attachment and proliferation commonly are used to evaluate viability. As the complexity of the desired cell function after thawing increases, so do the demands on post-thaw assessment. For example, post-thaw assessment of stem cells may require assay of membrane integrity as well as proliferation and the ability of the cells to differentiate into different lineages post-thaw. Post-thaw assays should be carefully developed and validated to avoid measurement bias. A certain fraction of cells will lyse during freezing, and methods of measuring cell recovery should include a complete assessment of cell losses (cells that have lysed as well as cells that are intact but not viable). Stability of cryopreserved cells can be assured by periodically thawing and testing a vial of the cells (also see ICH Q5D).

Testing for adventitious agents after the preparation of MCBs and WCBs should be routine, and *USP* general chapters *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩ and *Virology Test Methods* ⟨1237⟩ provide additional testing guidance (see also the FDA 2010 guidance cited in the *Appendix*). Testing for mycobacterial contamination, which may not be isolated in standard sterility testing, also can be considered for some cell substrates. Representative vials should be retrieved and tested for contamination (bacteria, fungi, *Mycoplasma*, and viruses). Numerous well-established methods are available for detecting *Mycoplasma* in cell cultures (see *USP* general chapter *Mycoplasma Tests* ⟨63⟩).

The batch record should be detailed, including the history of the cells and all activities starting from their receipt to release of the cell banks or products for use. The record should include detailed information about the cryopreservation process, including the procedure, the equipment used (with unique identifier), and a printed record of the freeze profile. The viabilities of the cryopreserved cells should be monitored over time to ascertain the effectiveness of the freezing process and the storage conditions. The information captured in each batch file of a cell line must be traceable to the original source, and all documents should be maintained and updated according to the quality management system in place.

CRYOPRESERVATION OF HUMAN CELL THERAPY PRODUCTS

Cell preservation methods are used to ensure product stability during hold, storage, and transport steps for a wide range of human cell-based products (additional information is found in ⟨1046⟩). Cells can be preserved in liquid suspension for up to a few days, but quantitative and qualitative changes in the cellular product invariably occur over time. Although preservation in the frozen state also affects the cellular product, it allows more predictable preservation of specific product characteristics over much longer time intervals.

For any given cell product, the decision to use cryopreservation depends primarily on the timing of final product administration in relationship to cell source collection and product manufacturing steps. Many patient-specific autologous and allogeneic products are maintained in liquid suspension, without cryopreservation, from starting cell source through final formulation and are released as fresh products after a relatively short time. However, many clinical applications require cryopreservation of the cell source, intermediate products, or final product. In these cases, cryopreservation can permit optimization of workflow during manufacturing, completion of lot-release testing, maintenance and management of a product inventory, transport of the product to the clinical site, and coordination of product administration with the patient's medical or surgical regimen. For example, umbilical cord blood is cryopreserved and stored in public banks for subsequent transport to, and temporary storage at, clinical transplant centers, where it is thawed immediately before infusion into a patient.

Development of a cryopreservation process for a clinical cell therapy must consider the consequences for the product, the patient, and the overall feasibility of the therapy. For the cell product, the manufacturer must ensure that expected cell losses because of cryopreservation and thaw occur in a manner that is reasonably predictable and must ensure that the final product administered to the patient will meet specifications for cell number, viability, and functional characteristics.

For the patient, cryopreservation may affect the efficacy and safety of the final product. For example, the cryoprotectant DMSO is associated with risk of predictable dose-dependent gastrointestinal, cardiovascular, and neurologic toxicities that typically are ascribed to histamine release. Residual DMSO in the final product should be estimated or measured. DMSO is categorized by ICH as a Class 3 (relatively low risk) solvent or excipient in pharmaceutical products, and amounts of up to 50 mg/day, or less, are considered acceptable without justification (see ICH Q3C). Cryopreserved cellular products frequently contain 10–20 times this amount unless they are washed after thawing. Even higher amounts of DMSO may occur with administration of multiple cryopreserved products, and this occurs commonly with autologous peripheral blood stem cell transplantation. A DMSO limit of 1 g/kg recipient weight/day is commonly used in clinical cell therapy practice. Procedures to prevent DMSO toxicity also should be considered. It is common clinical practice to premedicate patients with diphenhydramine or other antihistamine agents to prevent DMSO toxicity. Product washing methods by either manual (centrifugation) or automated methods also can be considered but must be validated to ensure adequate postwash recovery and cell function.

Use of cryopreserved cell therapy products may require the clinical site to receive, store, thaw, and perform other final preparation steps on the cryopreserved product. Feasibility assessment requires consideration of the site's capabilities with regard to specialized personnel, training, equipment, and facilities to execute those tasks.

If cryopreservation is planned as part of the manufacturing process, development teams must consider the effect of cryopreservation on cell number and characteristics and should require reliable methods for cell enumeration and assessment of cell viability and function. During development runs, more assays often are performed than will be required eventually for in-process and final product testing. This is done in order to evaluate the effects of each manipulation of the product. Because some cells may be more susceptible than others to freeze–thaw damage, these studies should include assessment of selective losses of important cell subpopulations within the product.

As described in the *Introduction,* several critical processes influence the outcome of a cryopreservation protocol. Following is a brief discussion of issues unique to cell therapies.

Prefreeze Processing

If cells are harvested with plasma present, samples should be properly processed with an anticoagulant to prevent clotting. In addition, some cells are prone to clumping or aggregation when centrifuged, and some cell products may exhibit excessive damage or loss of one or more populations within the product. Cells harvested from adherent or nonadherent culture may include substantial numbers of dead or fragile cells. Therefore, centrifugation and wash steps should be optimized and specific for the product's cellular contents, suspension volume, suspension medium, and container, along with appropriate evaluation of the cellular product before and after these manipulations. Prefreeze processing should not result in cells that are stressed (e.g., cells that demonstrate elevated early apoptotic markers or temperature-shock responses) before the start of the freezing process, or cell losses will be higher than expected.

Reagents and Containers

Clinical-grade reagents and containers should be used whenever possible (see *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩). It is customary to use sterile, single-use, disposable plastic bags or cryovials that have been qualified for the specific cryopreservation process and subsequent storage conditions. Cryopreservation media for cell therapy products usually consist of isotonic saline-based solutions with one or more CPA, typically the intracellular cryoprotectant DMSO at 5%–10% final concentration with or without an extracellular cryoprotectant such as hydroxyethyl starch. The use of human-derived protein additives such as human serum albumin, serum, or plasma is common, but they may need extensive qualification, so they should be avoided if possible and alternatives should be evaluated. Additives such as animal-derived heparin, citrate-based anticoagulants, and DNase sometimes are used. Many centers formulate their own cryopreservation media, but commercial cryopreservation media, which typically include 5%–10% DMSO and other proprietary components, increasingly are used by cell therapy manufacturers to eliminate variability and the need for additional qualification activities associated with local formulation.

General Chapters

Addition of Cryoprotectant Solution and Cooling

Procedures for introduction or removal of a cryopreservation solution should be assessed before freezing to ensure that cell losses resulting from this step are minimized. The cryopreservation medium usually is added to the cell suspension in steps or gradually (e.g., using a syringe pump) to prevent cell losses resulting from osmotic stress. It is common to prechill the cryopreservation medium and keep the cell suspension and the admixture chilled using cold packs, a frozen blanket, or a chilled work surface to prevent heat-related cell damage during addition of DMSO. Once the cryopreservation medium is added, the cell suspension typically is transferred to the precooled chamber of a controlled-rate freezer. During the freeze process, a record of chamber and product temperature over time, or freeze curve, is generated for inclusion in the production record. Product temperatures can be recorded from a probe placed on the product bag's outer surface or from the inside of a comparable product in a dummy bag or vial that undergoes concurrent freezing.

Storage and Transport

Cryopreserved cell therapy products typically are stored and transported at temperatures of −150° or colder. FDA requires screening and testing for evidence of transmissible disease only for allogeneic donors of cell therapy products and not for autologous donations. However, many centers test autologous donors as well and segregate products from autologous donors who are known to have transmissible diseases when their products must be stored. A report of hepatitis B cross-contamination of cellular products within a liquid-nitrogen storage tank led to the currently common practices of storage in the vapor phase of liquid nitrogen and the use of overwrap bags to reinforce product containment. Liquid-nitrogen vapor phase storage may be associated with vertical temperature gradients: products at the top of the storage tank may have a warmer temperature than those stored at the bottom of the tank. Temperature gradients should be monitored, and vertical temperature gradients should be minimized, e.g., by use of metal heat shunts. Overwrap bags may reduce the warming rate of the sample if used during the subsequent thaw process, and their use should be qualified as part of the overall cryopreservation process validation.

Transport of cryopreserved cell therapy products usually is accomplished by using dry shippers containing absorbent material that can be charged with liquid nitrogen to maintain vapor-phase temperatures for up to 2 weeks if properly charged. Data loggers are used to document the temperature history during transport. These shipping containers and procedures must be validated before cell therapy products for clinical use are shipped in the containers.

Warming (Thawing)

Although bedside thawing of cell therapy products before infusion has been a common clinical practice, the use of trained personnel in a controlled laboratory environment is now recognized as the preferred method for thawing because it allows a more standardized process and a higher degree of control when staff must respond to a container failure, which may require product salvage in a more sterile environment. Product thawing typically is done by immersion in 37° water baths and using overwrap bags to minimize product loss and contamination in case of primary container failure. Bags may be gently kneaded during thaw to reduce temperature gradients across the bag and to accelerate thawing. The product is removed from the water bath when some ice is still present in the product but the majority of the product is thawed.

Post-Thaw Processing

DMSO is toxic to cells in liquid suspension. Toxicity may be reduced by diluting or washing the cell suspension before infusion or further manipulation. Because cryopreserved, thawed cells are more sensitive to volumetric expansion when the cells transition from a hypertonic solution to an isotonic solution, the dilution and washing solutions and methods must be carefully designed and validated. Cell washing using either a conventional centrifuge or an automated device can result in additional mechanical stress to the cells, so cell losses must be assessed by an appropriate method before a specific method is implemented in clinical practice.

Quality Control Practices

Quality management of cryopreservation, storage, and thaw of clinical cell therapy products must incorporate quality system elements common to current Good Tissue Practices (cGTP) and current Good Manufacturing Practices (cGMP), including personnel qualification, facility controls, document control, control of equipment and materials, label control, and use of validated SOPs (21 CFR 1271, 210, and 211). Quality control practices specific to clinical cryopreservation typically include assessment and documentation of freeze curves for all products, retention of tubing segments and vials for subsequent testing, and regular monitoring of post-thaw product quality. Practices required by cGTPs and applicable to all human cell- and tissue-based products include measures to ensure accurate and complete labeling and records, to ensure that the correct product goes to the patient, and to allow tracking of the cell product from collection to infusion. The practical implications of these requirements are that labeling and records, including inventory systems, for cryopreserved products must be designed to pre-

vent errors in identification of products. Identity verifications of products moving into and out of cryopreserved storage are performed routinely, e.g., with two people checking the product label against records. If necessary for additional verification of product identity, the contents of a tubing segment attached to the product bag can be thawed and tested before thaw of the entire product. ISBT 128, an internationally recognized system for labeling of blood and cell-therapy products, incorporates use of consistent product nomenclature and barcoding of the product from donor source through administration (see *Appendix*).

HEMATOPOIETIC STEM CELLS

Studies of the response of hematopoietic stem cells (HSCs) to freezing began in the 1950s, and cryopreserved HSCs have been widely used in clinical practice during the past 30 years. The most common method of cryopreserving HSCs for clinical applications involves the use of 10% DMSO and a controlled-rate freezer set at a cooling rate of 1°/min. Another method, less commonly used, involves the use of passive freezing of the HSC product in a –80° mechanical freezer and the use of 5% DMSO + 6% hydroxyethyl starch solution. Cells commonly are frozen at densities of 30–50 × 10^6 cells/mL. Exceeding cytocrits of 20% (v/v) has been shown to reduce cell recovery. Post-thaw assessment of the sample consists of enumeration of nucleated cells, viable $CD34^+$ cells, and hematopoietic colony-forming units, along with subsequent calculation of recoveries from corresponding prefreeze values.

HSCs cryopreserved for clinical use may be obtained from bone marrow, mobilized peripheral blood, or umbilical cord blood (UCB). Each source has unique requirements for preservation. For example, peripheral blood progenitor cell products contain larger numbers of cells and may be frozen in multiple bags with relatively high cell concentrations. Protocols for UCB preservation may include the use of syringe pumps to introduce cryopreservation solutions while minimizing osmotic stresses. For UCB, specialized solutions often are used after thaw to dilute or remove DMSO while minimizing osmotic stress for the cells.

MESENCHYMAL STEM CELLS

Research on the clinical use of mesenchymal stem cells (MSC) has grown rapidly since the mid-1990s. Reliable, safe, and efficient methods of cryopreservation and storage are critical, especially for allogeneic off-the-shelf MSC products manufactured in multiple product doses for treatment of a large number of patients with a range of clinical indications.

Because MSCs traditionally have been generated in cultures that contain fetal bovine serum (FBS), cryopreservation media for these cells often have incorporated FBS. More recently, alternatives to gamma-irradiated FBS are being explored for the culture expansion before cryopreservation, and cryopreservation has been successful in media that contain 5%–10% DMSO and other components without bovine sources of protein. Although there is no consistent method of post-thaw cell processing, some protocols include dilution or washing of the cells to mitigate the effects of DMSO. Emerging clinical applications for MSCs may require repeat dosing of the cellular product, a practice that requires attention to potential immunogenicity of components of the cryopreservation medium, e.g., FBS or other proteins.

Post-thaw assessment of MSCs typically has involved the use of membrane-integrity dyes such as trypan blue, surface antigen expression, and evidence that the cells are capable of multilineage differentiation. Because the mechanism of action of MSCs may involve the immunomodulatory or trophic properties of the cells, post-thaw assessment also should include relevant cell function.

LYMPHOID CELLS

Lymphocytes are used for a variety of clinical applications including immunotherapy to treat cancer, viral infection, and autoimmune disease. Therapy based on lymphocytes may consist of mixed lymphocyte populations or lymphocyte subpopulations that have been selected or activated ex vivo, e.g., regulatory T cells, natural killer cells, and activated T cells. As with hematopoietic cells, lymphocytes typically are cryopreserved using a 10% DMSO solution and a controlled cooling rate of 1°/min.

Lymphocytes may undergo extensive post-thaw apoptosis, which can influence the clinical efficacy of the cells. Highly purified populations of lymphocytes may exhibit higher levels of post-thaw apoptosis than mixed lymphocyte populations. Strategies such as caspase inhibition and cytokine rescue have been used to diminish post-thaw apoptosis of lymphocytes.

PRESERVATION OF HUMAN PLURIPOTENT STEM CELL LINES

Pluripotent stem cells (PSCs) are cells that appear to have the capacity to (1) undergo self-renewal and replicate indefinitely and (2) generate cells that are representative of the three germ-layer tissues required to create all cells of the human body as demonstrated by the capacity to generate teratomas in immune-deficient mice. The two predominant types of stem cell line used for in vitro laboratory research are human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). hESCs are derived from donated surplus blastocysts by isolation and culture of the blastocyst inner cell mass (the tissue that would have progressed to form the embryo). hiPSCs are created by artificial reprogramming of somatic cells (by the

delivery of reprogramming factors using a variety of methods) to yield cells that express the critical properties of PSCs listed above. hiPSCs can be derived from a range of somatic cell types using an increasing range of methods to secure expression of certain reprogramming factors. A range of tissue-derived cultures are known to harbor stem cell populations in vitro (e.g., subcutaneous fat, bone marrow, cord and blood of the fetal umbilicus, primordial germ cells from the fetal neural crest, and neural stem cell spheroid cultures) that have been shown to have a limited capacity for in vitro replication and have not been established as stable diploid PSC lines. Such cultures will not be covered in this chapter, and the following sections will deal specifically with hESC and hiPSC lines.

PSC lines are complex multicomponent cell cultures and may contain a variety of different cell populations with greater or lesser degrees of differentiation or lineage commitment. However, the key property of a stem cell culture, self-renewal, must be sustained. In addition, the cells must retain the capacity to undergo asymmetric division to yield two different daughter cells: one that is a stem cell identical to the parent cell and one with a reduced degree of potency (i.e., ability to generate different cell lineages).

Development of Current Methodologies for PSC

Currently, individual labs have their preferred methodology, no single approach appears to dominate for routine use, and it seems possible to obtain acceptable levels of post-thaw viability and recovery by either vitrification or controlled slow rate cryopreservation; however, a common method that results in better cell recovery is described here. Briefly, this technique involves placing colonies of hESCs in a vitrification solution composed of 20% DMSO + 20% ethylene glycol (EG) + 0.5 mol/L sucrose after equilibration with lower-concentration DMSO + EG solutions. The colonies are loaded into straws and plunged into liquid nitrogen. Another common method involves placing colonies in a solution consisting of 10% (v/v) DMSO and using either a passive freezing device or a controlled-rate freezer designed to achieve an average cooling rate of approximately 1°/min.

Vitrified IPS or hES samples have special storage and shipping requirements. All cryopreserved or vitrified samples must be stored at temperatures below the glass transition temperature of the sample, and for vitrified samples this is much lower (e.g., −150°) than for traditionally cryopreserved cell samples. In addition, fluctuations in temperatures during storage or shipment can lead to crystallization and therefore degradation of the sample. Because vitrified samples are sensitive to these temperature fluctuations, they should be shipped at liquid nitrogen temperatures and should not be shipped on dry ice.

Challenges for the reliable and reproducible preservation of PSC lines lie not just in the preservation process itself but also in the preparation, cryoprotection, and recovery procedures. Specifically, methods of harvesting and handling before cryopreservation may result in significant cell losses. Processing procedures should be made as reproducible as possible and with the use of an SOP to enhance the reproducibility of preservation outcomes. Furthermore, the effective banking of PSC lines is challenged by the methods by which the cells typically are passaged and harvested for preservation (i.e., individual colonies dissected and transferred as small colony fragments to fresh culture flasks for expansion or to preservation medium for freezing). First, in order to avoid extensive loss of viability in the earlier harvested cells, cultures used to make a bank actually may require preservation in small batches over a working day. Second, preparation of PSC banks comprising smaller pools of cell colony fragments is not only time consuming and laborious, but it also makes it impossible to homogenize the preparation of cells before aliquoting into vials, as would happen with more traditional methods of preserving cell lines. Thus, consistency between vials of PSC lines is compromised. Third, freezing PSCs as colony fragments preserves the gap junctions known to form between cells, potentially leading to intercellular propagation of ice crystals and extensive loss of viability.

To avoid some of these issues, analysts can use enzymatic disaggregation of colonies to simplify and accelerate cell harvesting and to enable cryopreservation of more homogenous, single-cell suspensions before preservation. To be prudent, some laboratories choose to perform enzymatic disaggregation solely before cryopreservation but not for routine culture. Whenever possible, analysts should use non–animal-sourced enzymatic agents for cell dissociation. Another important characteristic of PSC lines is the common but variable incidence of undirected differentiation that occurs within colonies and may vary considerably across the many colonies in a culture. Colonies with a high proportion of differentiated cells should be discarded because the differentiated cells are an undesirable component of a PSC culture and may affect the properties of the undifferentiated cells within the colony.

Points to Consider in the Preservation of PSC Lines

Analysts should consider a number of important factors in the preservation of PSC lines that should be addressed at the levels of preparation, harvesting, banking, and testing of cryopreserved stocks.

EVALUATION AND HARVESTING

Analysts should observe cell cultures on a regular basis. Cultures that exhibit high levels of differentiated cells should be discarded. An individual investigator or banking facility should have a quality control program that evaluates differentiation of cultures on a regular basis and develops threshold levels of acceptable differentiation in a culture. The protocol for harvesting followed by equilibration of the sample with CPAs should be carried out rapidly in order to minimize cell losses because of harvesting.

General Chapters

THE BANKING PROCESS

Conventional cell banking procedures require the development of MCB and WCB. Cells in the MCB should be preserved at an early passage number (P10–P20) in order for experimental and cell line development work to be performed on cells at the lowest possible passage number. The first WCB should be established with the minimum passages to achieve the cell number required. To promote consistency, the WCBs subsequently can be replaced at the same passage level from the MCB as required.

VIABILITY

Determination of post-thaw viability and function for PSCs can be complicated and is often done incorrectly. The most common method of post-thaw assessment is use of a membrane integrity dye. As a measure of viability, the fraction of cells with intact membranes is compared to those without. Another fairly common method is to quantitate PSC colony formations. The total number of colonies seeded into a plate is counted and then is counted again after a certain incubation time. This method also evaluates other functional characteristics of the cells, such as their ability to attach and proliferate. Note that procedures such as harvesting and cryopreservation may induce apoptosis. Staining for early (phosphatidylserine on the cell surface) or later (Annexin VI) apoptotic markers may provide insights into the general health of frozen and thawed cultures.

HOMOGENEITY

As already described, preservation of colony fragments by vitrification can exacerbate the lack of homogeneity between vials or straws of cells. When larger banks of PSCs are established, analysts should test vials from early, middle, and late positions in the filling sequence of the cell bank for viability, growth rate, and key markers as indicated in *Viability*.

STEMNESS

In order to check that a PSC line has not lost any of its stem cell characteristics during preservation, analysts should check expression of a number of key stem cell-related markers. One extensive study analyzed 59 hESC lines and a panel of 94 genes and resolved five stem cell-related molecules for which mRNA was expressed consistently in hESCs. These genes now are included in commercially available microfluidics gene cards that are specifically designed for investigating stem cell populations. If it is crucial to demonstrate that the PSCs have retained their pluripotency, then a number of characterization tests can be performed, including teratoma formation in immune-deficient mice, formation of trilaminar embryoid bodies, and directed differentiation to demonstrate that the culture can produce representatives of each of the three germ layer tissues that are required to form all of the cells of the human body.

GENETIC STABILITY

In both iPSC and hESC lines, it is not unusual for clones of abnormal karyotype to arise on extended passage and overgrow the culture. Thus analysts should monitor cultures for such abnormal cells. Traditionally, this has been performed by karyotypic studies of metaphase spreads of the cells using Giemsa staining. The occurrence of nondiploid cells, even at very low incidence, can be problematic. Guidance documents such as the 2009 International Stem Cell Banking Initiative are helpful to determine if such cultures should be discarded. However, newer procedures such as array comparative genome hybridization and single-nucleotide polymorphism arrays provide much more detailed analysis of genetic stability and can be used in parallel with Giemsa banding to give greater confidence in genetic stability.

BEST PRACTICES

In addition to following good cell culture practices, analysts should note the availability of a specific guidance that contains principles and best practices in the procurement, banking, testing, and storage of hESCs for research purposes (see the ISCB 2009 reference in the *Appendix*). This guidance is useful for both iPSC and hESC lines.

CELL SUBSTRATES USED IN PRODUCTION AND CHARACTERIZATION OF BIOTECHNOLOGY-DERIVED AND BIOLOGIC THERAPEUTIC PRODUCTS

A wide variety of both recombinant and nonrecombinant cells are cryopreserved and used in the production and characterization of human biologics and biotechnology-derived (B&B) products (more information can be found in ICH Q5D *Derivation and Characterisation of Cell Substrates Used for Biotechnological/Biological Products)*. The major groups include cell lines derived from mammals (including humans), insects (primarily moths), and selected strains of bacteria and yeast. The most common microbial substrates used for production of human biotechnology-derived products are recombinant strains of *Escherichia coli, Pichia pastoris,* or *Saccharomyces* spp. (yeasts). Despite the high degree of diversity among cell types used to manufacture B&B

products, there is a surprising degree of uniformity across cryopreservation practices and the same principles apply to cells used for manufacture and those used in tests for adventitious agents and product potency.

Typically, a two- or three-tiered cell banking system is used for maintenance of manufacturing cell lines or microbial strains. For those that use a three-tier system, the first tier bank can be referred to as a research, seed, stock, accession, pre-MCB, or parent cell bank. The source of the pre-MCB can be a research or development laboratory, or cells can be purchased from a commercial repository. It is advisable to characterize the parental cell bank prior to its use in cloning. The second tier (or first tier in a two-bank system) MCB or Master Cell Stock (MCS) is prepared directly from this parent cell bank with minimal cell passages or generations. The MCB or MCS is extensively tested to confirm purity, phenotype, genotype, protein expression, or other important attributes. The WCB or Working Cell Stock (WCS) is derived from vials of the MCB after successive passages in culture. The WCB is the manufacturing cell substrate that is scaled up through repeated subcultures to seed the final production bioreactor, fermenter, or lot of culture vessels (e.g., roller bottles). At each tier in the cell banking system, proper cryopreservation is paramount to success in both product development and manufacturing. In some cases, the end of production cells also may be banked for testing purposes as part of cell bank qualification.

Mammalian and Insect Cell Lines

Mammalian cell lines are the cellular substrates of choice for the production of complex protein molecules. Mammalian cells possess the intrinsic biological machinery required for posttranslational glycosylation of proteins that often is critical for stability and bioactivity in humans. Both diploid and heteroploid cell lines (including hybridomas) are used for production of biotechnology-derived therapeutics. Diploid cell lines are common vaccine substrates [e.g., WI-38 and MRC-5 (human fibroblast cell lines), BHK-21 (baby hamster kidney cell line), and MDCK (Madin-Darby canine kidney)]. Additionally, the African green monkey Vero cell line (a heteroploid cell line) is used for several US-licensed vaccines. Today, commonly used heteroploid cell lines include various recombinant Chinese Hamster Ovary and human embryonic kidney cell lines. Cryopreservation methods are fairly well standardized for these cell lines. However, investigators may find it useful to investigate the toxicity of different cryoprotectants and concentrations when they use a new cell substrate.

Insect cell lines have proven their capability for production of various recombinant polypeptides. The most common cell lines are derived from the moths *Spodotera frugiperda* and *Trichoplusia ni,* and their established cell lines are called Sf9 and Tn5 (or High Five), respectively. Production of recombinant proteins employs recombinant baculovirus infection for transfer of heterologous genes. Although insect cell lines require different nutritional factors, lower incubation temperatures, and higher osmolarity than their mammalian counterparts, the same essential elements of cryopreservation apply to both groups.

In addition to the guidance given in the *Key Elements of Cryopreservation Practice* section, these additional points should be considered for animal cell line substrates:

PREFREEZE PROCESSING

Analysts should ensure that cells are not contaminated and are still viable. For diploid cells, analysts should grow to a passage level to maintain diploidy and below the intended level for use. Analysts pool cell cultures and perform a cell count to determine the number of viable cells available for banking. Harvested cells are centrifuged at a relatively low speed for a short duration, e.g., 100–200 × *g* for 5–10 min, preferably using a refrigerated centrifuge.

CPAS AND CRYOCONTAINERS

The membrane-permeable CPA of choice is 5%–10% (v/v) DMSO diluted in fresh growth medium. For certain sensitive cell lines, cell culture-conditioned medium can be added to supplement the cryopreservation medium. DMSO must be sterile and tissue-culture grade (>99% purity). The most appropriate cryocontainer is a presterilized polypropylene screw-cap vial designed for cryogenic storage in vapor phase liquid nitrogen.

INTRODUCTION OF CRYOPRESERVATION MEDIUM

Immediately following centrifugation of cells, growth medium is removed from cell pellets, and cells are gently resuspended by slow addition of cryopreservation medium that is often precooled for many cell types. The cell suspension is immediately diluted with an appropriate volume of cryopreservation medium based on viable cell count and targeted cell density (typically, cell banks are produced at a viable cell density of approximately 1×10^7 cells/mL). As mentioned in the previous section, cryopreservation medium is highly hypertonic, and exposure time should be limited. The final cell suspension is transferred to a vessel in which the cells can be gently mixed during vial filling to facilitate uniformity of the cell bank.

COOLING AND CONTROLLED FREEZING AND STORAGE

Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine. In either case, vials are usually refrigerated as the filling progresses in order to minimize potential toxic effects of DMSO at higher tem-

peratures. Time limits should be established for the entire filling process and recorded in the batch record. Immediately after the vials are filled, analysts should transfer them into a controlled-rate freezer or, alternatively, into an ultracold static freezer (e.g., −80°) using an insulated container designed for controlled cooling. Any controlled freezing system should be properly qualified to ensure expected cooling rates are delivered to all vials within the load. When using a controlled-rate freezer, analysts determine optimal cooling programs empirically, but a rate in the range of 1°–5°/min after transition through heat of fusion should be acceptable for most cell lines (colder than about −40°, the rate can be increased, e.g., 10°/min). After vials have been frozen to approximately −80° or below in the freezing system, they are transferred as quickly as possible to liquid-nitrogen vapor phase storage units.

WARMING AND VIAL THAWING

In general, cells frozen at a slow cooling rate should be thawed as quickly as possible to maximize cell viability. In order to ensure uniformity of temperature, vials should be transferred directly from liquid-nitrogen vapor phase storage into a portable vapor phase (dry) shipper for transport to the laboratory. If a liquid-nitrogen Dewar is not available, then vials can sometimes be packed in dry ice, but this process should be demonstrated as suitable because it can result in detrimental pH changes. After transfer to the laboratory, vials are placed directly into a warm water bath (e.g., 37°, making sure caps are not immersed), a bead bath, or a thermoblock. Note that insect cell lines should be thawed at 27°–30°. Vials should be agitated to facilitate uniform thawing of cells. Immediately after thawing, vials should be transferred into the biological safety cabinet and sanitized before opening. Typically, growth medium is slowly added to thawed cells while agitating to dilute DMSO and to slowly reduce the osmolarity of the post-thaw milieu back to a physiological level.

POST-THAW PROCESSING

Different methods can accomplish this step, but analysts should achieve sufficient dilution of the DMSO and return the cells to their normal isotonic growth environment. Manipulation of cells immediately post-thaw should be minimized because of the stresses induced by the freeze–thaw process. For example, pipetting and centrifugation should be minimized.

MICROBIAL STRAINS: *E. COLI*, YEAST, AND BACTERIAL VACCINE STRAINS

Recombinant *E. coli* strains have a proven track record for production of a number of commercially viable biotechnology-derived products, including recombinant insulin analogs, human growth hormone, and parathyroid hormone. Compared to their mammalian and insect counterparts, recombinant *E. coli* strains are relatively simple to grow and scale up to large volumes, e.g., 40,000 L. However, bacteria lack the sophisticated cellular machinery for building more complex protein molecules that require post-translational modifications such as glycosylation. Yeasts are unicellular, eukaryotic cells that can be manipulated genetically to produce a wide range of recombinant proteins and peptides with limited complexity. Despite the evolutionary distances between the mammalian and microbial cell substrates, the same set of essential cryopreservation elements described above apply, with the following unique points:

PREFREEZE PROCESSING

Cultures should be propagated in shake flasks to late logarithmic or early stationary phase using strain-specific growth conditions.

CPAS AND CRYOCONTAINERS

The cell wall or cell membrane-permeable CPA of choice is glycerol (although DMSO is also sometimes used) at concentrations typically ranging from 5%–10% (v/v). Synthetic glycerol should be used for registration of commercial products along with other raw materials that are free of animal components.

INTRODUCTION OF CRYOPRESERVATION MEDIUM

Because microbial cells possess cell walls for protection and support of cytoplasmic contents, physical manipulation and osmotic shifts do not have the same negative impact seen in animal cell lines. Immediately after centrifugation, cell pellets are vigorously resuspended in the cryopreservation medium (to a dilution based on requirements of the cell bank for number of vials and viable colony-forming units/mL). This dilution step can be based on active measurement of culture optical density or other cell enumeration assays. The final cell suspension is transferred to a vessel in which the cells can be mixed during filling of final containers to facilitate uniformity of the cell bank. Because of potential toxicity, analysts should limit the time of exposure to glycerol, despite the relative robustness of microbial cells. Vials can be filled manually by using a hand-held pipetting device or by using an automated vial-filling machine.

COOLING AND CONTROLLED FREEZING AND STORAGE

Again, because of their inherent robustness, microbial cells do not require as strict control of cooling rate as do animal cell lines. Consequently, the choice of freezing system or method has a smaller effect on the viability of the cell bank. Filled cryocontainers simply can be transferred into an approximately −80° freezer overnight followed by transfer to vapor phase liquid nitrogen. Alternatively, microbial cell banks may not use liquid nitrogen storage but can be stored at approximately −80°. However, if liquid-nitrogen storage is available, it is preferred for long-term storage (e.g., years). If a controlled-rate freezer is employed, then it should be fully qualified to deliver a uniform cooling rate that is within an expected range, e.g., 1°–5°/min.

WARMING AND CONTAINER THAWING

Controlling the rates of cooling and warming is less critical for microbial cells, but the cells still should be thawed as quickly as possible using a warm water bath (e.g., 30°–35°). Temperatures can be adjusted to the incubation temperature of the strain.

POST-THAW PROCESSING

Growth medium is added to dilute the thawed cells to a desired level of colony-forming units/mL and to induce removal of intracellular glycerol.

All cells are not created equal and are divergent by nature. A broad span of evolutionary time separates the various cell substrates used for manufacture of human B&B products. Fortunately for the practitioner of industrial cell culture, cryopreservation strategies converge to a set of shared principles and methods that translate across evolutionary paths.

APPENDIX

Useful Guidances

- FDA. Guidance for industry: characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications. 2010. http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf. Accessed 24 September 2012.
- ICCBBA. OECD best practices guidelines for BRCs [128 Standard Technical Specifications]. 2007. http://www.iccbba.org/. Accessed 01 October 2012.
- ICH. Q5D Derivation and characterisation of cell substrates used for biotechnological/biological products. 1997. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5D/Step4/Q5D_Guideline.pdf. Accessed 25 July 2013.
- Coecke S, Balls M, Bowe G, et al. (2005). Guidance on good cell culture practice. A report of the second ECVAM Task Force on Good Cell Culture Practice, *Altern Lab Anim.* 2005; 33(3):261–287.
- Andrews PW, Arias-Diaz J, Auerbach J, et al. Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes: International Stem Cell Banking Initiative. *Stem Cell Rev.* 2009; 5(4):301–314.
- WHO. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for characterization of cell banks. 2010. http://www.who.int/biologicals/Cell_Substrates_clean_version_18_April.pdf. Accessed 26 July 2013.

〈1046〉 CELLULAR AND TISSUE-BASED PRODUCTS

INTRODUCTION

This general chapter provides a comprehensive overview of considerations for the development of cellular and tissue-based products. A collection of terms commonly used in this field is provided under *Glossary and Definition of Terms*. Cell and tissue-based therapies are medical products that contain human or animal cells that will be administered to humans to repair, replace, regenerate, or augment a recipient's cells, tissue, or organs that are diseased, dysfunctional, or injured. The source cells or tissue can be harvested for use without manipulation or may be propagated, expanded, pharmacologically treated, or otherwise altered in biological characteristics ex vivo before administration. The diversity of clinical indications and types of cell and tissue-based products are shown in *Table 1*.

Table 1. Examples of Cell-Based Therapy Products

Indication	Product
Hematopoietic stem cell transplantation following ablative therapy	Hematopoietic stem and progenitor cells that have been harvested, propagated, selected, and/or treated for removal of contaminating cells by means of devices and/or reagents
Cancer	T cells, NK cells, dendritic cells, or macrophages exposed to cancer-specific peptides to elicit an anticancer response; autologous or allogeneic cancer cells, genetically or biochemically modified and irradiated to elicit an anticancer response
Diabetes	Encapsulated β -islet cells
Myocardial infarction	Autologous or allogeneic stem/progenitor cells; skeletal myocytes; cardiac-derived stem cells
Graft-versus-host disease	Allogeneic mesenchymal stem cells
Wound healing	Autologous keratinocytes or allogeneic dermal fibroblasts on a biocompatible scaffold
Focal defects in knee cartilage	Autologous or allogeneic chondrocytes with or without a biocompatible scaffold
Bone repair	Mesenchymal stem cells in a biocompatible scaffold
Neurodegenerative diseases	Neuronal progenitor cells derived from embryonic, fetal, or adult source tissues; cells genetically modified to secrete neurotrophic factors, with or without encapsulation
Infectious disease	Activated T-cells
Autoimmune disease	Regulatory T-cells (T_{reg})
Spinal cord injury	Neuronal progenitor cells
Organ repair or regeneration	Autologous or allogeneic cells on biocompatible biomaterials (gels) or 3-dimensional scaffold structure

Cell therapy products can be modified by treatment with integrating or nonintegrating genetic materials (DNA, RNA, siRNA, etc.) so that the pattern of gene expression is changed. Typically, cells are taken from the patient and are modified outside of the body before they are returned to the patient. Regulatory bodies consider the ex vivo gene-modified cellular product to be a gene therapy product. A great deal of information in this general chapter is relevant to processing, characterization, manufacturing, and administration of genetically modified cells. However detailed information about the use of various gene transfer systems, patient monitoring considerations, genetic analysis, and other issues pertinent to gene therapy products are addressed in *Gene Therapy Products* ⟨1047⟩.

This general chapter describes issues related to the manufacturing, sourcing of components, and characterization of cellular or tissue-based products to ensure their safety and efficacy. A list of relevant regulatory and guidance documents is presented in the *Appendix*. Manufacturers of cellular or tissue-based products should consider and apply the controls and procedures outlined in this chapter to ensure the products' safe use in humans. New methodologies are continually being developed and validated and will be included in the United States Pharmacopeia (*USP*) as they become available. *USP* monographs for specific tissue and tissue-based products outline test specifications that should be met throughout a product's time in the market place. The term cellular product refers to living human or animal cells or tissues that have been manipulated or are used in ways that result in their regulation as somatic cellular therapies, as defined by the US Food and Drug Administration (FDA). A *tissue-based product* refers to human tissues subject to regulation under good tissue practices (GTPs). *Combination products* refer to cells combined with medical devices, such as a natural or synthetic scaffold.

Considerations for Incorporating Quality System Concepts Early in Cellular and Tissue-Based Product Development

Current and future regulatory requirements will continue to challenge developers of cellular and tissue-based products to incorporate robust quality attributes early in the design phase to ensure a focus on patient safety by means of a high degree of process understanding. Modern quality systems that harmonize current Good Manufacturing Practices (cGMPs) with other non-US pharmaceutical regulatory systems [such as the International Conference on Harmonization (ICH) and the International Organization for Standardization (ISO)] and the FDA medical device quality system are being recognized as the new global standards. These new standards include product development concepts such as Quality by Design (QbD) and Process Analytical Technology (PAT). Moreover, these quality systems integrate approaches to continual improvement and risk management that promote adoption of the latest scientific advances and innovative manufacturing technologies.

Employing the principles of Quality Risk Management (QRM) early in product development may identify areas of risk that can be mitigated before they are incorporated into the manufacturing process and affect the safety and efficacy of the product. Developers of cell and tissue-based products should employ risk management and assessment techniques as a key component of their quality systems. *Risk management* is defined as a systematic process for the identification, assessment, and control of risks to the quality of the cell or tissue-based product across the product lifecycle. Using QRM techniques can help achieve

safe and efficacious products by assessing patient risks, determining design space boundaries, or ranking quality attributes. QRM can also establish and maintain a state of control by using risk management to drive process control. Finally, QRM can be used to facilitate continual improvement by prioritizing opportunities for improvement. The level of effort, formality, and documentation of the risk management process should be commensurate with the level of risk, should be based on scientific knowledge, and ultimately should be linked to patient protection.

The elements of risk management have become an accepted paradigm and can be readily accessed in FDA and international regulatory guidance documents, especially ICH Q9. A number of tools have been developed to facilitate this assessment. These tools provide a quantifiable means of prioritizing risk so that higher-risk elements of a process can be identified and corrected.

Depending on the objective of the risk management program, risk analysis can be more or less formalized. Preliminary, less formal risk analysis comes into play when a risk assessment needs to be expedited, as in the resolution of a manufacturing nonconformance. A more formalized risk assessment system is necessary for process or product development. This is especially important when limited resources must be prioritized. Formalized systems are predicated on well-established tools that can quantify risk in every phase or step of manufacturing. These systems can also be used in evaluating raw material choices, validation prioritization, facility alterations, equipment changes, and utility deliberations.

Formal risk analysis tools include process mapping, preliminary hazard analysis, Hazard Analysis of Critical Control Points (HACCP), Hazard Operability Analysis (HAZOP), Fault Tree Analysis (FTA), Failure Mode Effects Analysis (FMEA), and Failure Mode Effects and Criticality Analysis (FMECA).

For cell and tissue-based products, FMEA has been commonly used to identify, quantify, and prioritize risk. FMEA can assign a numerical rating in one of three categories:

- *Severity*, which is the consequence of a failure;
- *Occurrence*, which is the likelihood of the failure happening based on past experience or nonconformance; and
- *Detection*, based on the ability to detect the failure.

Each category is assigned a numerical rating (typically 1 to 5 or 1 to 10) consistent with the severity of the excursion from the operating parameter range, the probability of an excursion, and the likelihood of detecting an excursion before it has an effect on the product. Lower numbers refer to an unlikely probability of detection whereas higher numbers refer to the likelihood of a failure or hazardous effect. The product of the severity, occurrence, and detection values is a Risk Priority Number (RPN). In the risk-evaluation process RPNs are prioritized, and the most immediate remediation can be directed to areas of highest risk.

COMPONENTS USED IN CELL AND TISSUE-BASED PRODUCT MANUFACTURING

Introduction

Manufacturers of cellular or tissue-based products must ensure that all components used in manufacturing are appropriately qualified. Examples of components used in the production of cellular or tissue-based therapies include the source cells and tissues; natural or synthetic biomaterials; ancillary materials required during manufacturing but not intended to be present in the final therapeutic product; and excipients used in the formulation of cellular or tissue-based therapies.

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific component to ensure quality. The diversity of cellular and tissue-therapy products and of the materials used to produce them makes it difficult to recommend specific tests or protocols for a qualification program. Therefore, rational and scientifically sound programs must be developed for each component.

Material qualification activities will change as products move from clinical trials to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety concerns should be the primary focus of a material qualification plan. In the later stages, material qualification activities should be completely developed and should comply with cGMP.

Qualification of Source Cells and Tissues

Various human- and animal-derived cells and tissues serve as source material for cell and tissue-based products. Three sources of donor cells for cell-therapy products include:

1. The patient's own cells (autologous cell products)
2. Cells from another human being (allogeneic cell products)
3. Cells derived from animals (xenogeneic cell products)

The source of cells used for a particular cell or tissue-based therapy largely depends on compatibility, purity, and availability. Use of autologous cells has the advantage of minimal concerns regarding immune rejection. However, an autologous source is not always available and appropriate if the cell type is dysfunctional, malignant, not readily obtainable, or contaminated.

The alternative is a compatible allogeneic cell source that may be more readily available. Of primary concern with the use of allogeneic cell sources is immune incompatibility, which could lead to rejection of the administered cell or tissue-based therapy. In immunocompromised recipients, the donor cells may react to the patient's cells, leading to graft-versus-host disease, which can be life threatening. Despite the potential complications of using allogeneic donor cells or tissues, in the absence of other alternatives the risk-to-benefit ratio is often favorable. A number of approaches successfully circumvent immune barriers for the use of allogeneic sources. Immunosuppressive drugs developed for solid organ transplantation and advances in induc-

ing immune tolerance are increasingly applied to cell transplantation. Certain allogeneic cells elicit minimal immune reactions, even in HLA-mismatched recipients. Examples include mesenchymal stem cells, certain dermal and epidermal cells, and fibroblasts.

Despite advances in the derivation of new types of therapeutic cells, particularly stem cells (adult, fetal, embryonic and induced pluripotent cells), the ability to generate certain types of cells or tissues remains elusive. As a result, ongoing efforts use xenogeneic cells and tissues to treat a number of human diseases or conditions. Use of xenogeneic cells must address concerns about both immune rejection and transmission of animal viruses to humans (see *Animal Sources of Cells and Tissues*, below).

Some general principles in the sourcing of tissues include: (1) systems must allow the material to be traced back to the donor, while adhering to privacy legislation; (2) steps must be taken to prevent the transmission of infectious diseases from the donor to the recipient; and (3) aseptic procurement and processing must ensure the safety of the final product because terminal sterilization of products containing living cells and tissues is not possible. FDA has promulgated a specific set of regulations, referred to as GTPs, that specifically address the need to procure and process tissues in a manner that avoids transmission of a communicable disease. GTPs and/or GMPs must be followed for cell or tissue-based therapy products, depending on cell source and place in the product life cycle.

DONOR ELIGIBILITY

FDA has enacted a comprehensive set of regulations governing human tissues and human cells that are intended for implantation, transplantation, infusion, or transfer into a human recipient. These materials are referred to as human cells, tissues, or cellular or tissue-based products (HCT/Ps). Paramount for procurement of HCT/Ps for medical use is adherence to donor eligibility requirements. These dictate that a donor's relevant medical records must be reviewed to evaluate risk factors and clinical evidence of communicable disease agents. This includes obtaining a health history and performing a physical examination on a donor to screen for communicable diseases. In addition, donors must also undergo appropriate laboratory testing using FDA-cleared or -approved test kits for specific relevant communicable disease agents and diseases (RCDADs). Required disease testing will expand as new RCDADs are identified and FDA-approved or -cleared test kits become available. Two sources for information about communicable disease testing are FDA's *Guidance on Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products and AABB's Circular of Information* (http://www.aabb.org/Content/About_Blood/Circulars_of_Information/aabb_coi.htm). Donor eligibility determination is not required for autologous HCT/Ps.

HUMAN CELLS, TISSUES, OR CELLULAR OR TISSUE-BASED PRODUCTS

HCT/Ps may be sourced from normal healthy donors, cadaveric donors, or patients with diseases such as cancer. The suitability of tissue sourced from patients with cancer and other diseases should be assessed before collection to ensure adequate safety and function of the final cell therapy product. Additionally, the regulations in 45 CFR Part 46 apply to all federally supported human subject research. These regulations require that an Institutional Review Board review and approve the use of any tissue taken from a human donor. The regulations also include special considerations for research on prisoners, children, pregnant women, or gestational tissue. In all cases appropriate written consent must be obtained from the donor or the donor's next of kin describing the tissue that is being procured and its intended use.

The risk of disease transmission to the manufacturing operator should be minimized by appropriate training for handling potentially infectious materials and by the use of protective equipment and clothing. Tissues should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery.

Hematopoietic progenitor cells (HPCs) are one of the most extensively used cell sources for human transplantation. These cells can be collected from the bone marrow, peripheral blood, or umbilical cord blood. The source of cells depends on the patient, the disease, and the clinical protocol. Regardless of the cell source, methods for processing the cells are similar. HPCs can be sourced from healthy donors or patients with hematological disorders. In addition to FDA's HCT/Ps regulations, applicable guidelines and standards for the collection and processing of these materials have been published by the American Association of Blood Banks (AABB), the Foundation for the Accreditation of Hematopoietic Cell Therapy, and the National Marrow Donor Program (NMDP).

For cell or tissue sources obtained from surgical specimens or cadaveric donors, standard hospital operating room practices are applicable. The air quality in a typical limited-access operating room is adequate for such procedures. Procurement personnel must be appropriately trained in all aspects of tissue recovery, such as surgical scrubbing, gowning, operating room behavior, anatomy, surgical site preparation, and aseptic technique. Special care is required when tissue or organ procurement requires extensive manipulation of the bowel, which may result in the inadvertent puncture of the bowel. Tissue that contains microbial flora (e.g., skin) at the time of procurement can be adequately disinfected with antimicrobial or bactericidal agents and extensive scrubbing.

ANIMAL SOURCES OF CELLS AND TISSUE

Ideally, cellular therapy products would consist of human cells manufactured with minimal exposure to animal-based materials. However, at present important unmet medical needs may potentially be addressed by cellular therapy products from ani-

mal cells or tissues. One example is pancreatic islets intended to treat diabetes. Human sources of pancreatic islets are available only from pancreas donated at the time of death. The quality of donor organ islets is variable, and the available supply is inadequate to meet potential demand. One approach is procurement of pancreatic islets from appropriately qualified animal sources for subsequent use in humans (xenotransplantation).

Developers who intend to use animal cells or tissues in a cellular therapy product must adequately address public health concerns and must develop approaches to mitigate the potential risk of introduction and propagation of zoonotic infectious agents into the general human population. The *PHS Guideline on Infectious Disease Issues in Xenotransplantation* (January 2001) describes potential risks. The FDA guidance *Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans* (April 2003) reflects updated approaches and expectations to minimize risks of xenogeneic cellular products.

The use of animal tissue in the manufacture of cell therapy products requires that the tissue be sourced in a controlled and documented manner from designated pathogen-free animals bred and raised in captivity in countries or geographic regions that have appropriate disease prevention and control systems. In addition, the care and use of animals should be approved by a certified institutional animal care and use committee. Donor animals must have documented lineage, be obtained from closed herds or colonies, and be under health maintenance and monitoring programs. The animal housing facility should be USDA certified (large vertebrate animals) or Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) certified (small vertebrate animals) and should meet the recommendations stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), which can be obtained from AAALAC (www.aaalac.org). Such facilities should be staffed with veterinarians and other trained personnel who ensure animal health and disease prevention. The facility's procedures should be documented, and records should be kept. Health maintenance and monitoring programs should be based on standard veterinary care for the species, including physical examinations, monitoring, laboratory diagnostic tests, and vaccinations. A stepwise *batch* or *all-in–all-out* method of source animal movement through the facility can minimize the potential for transmission of infectious agents.

Feed components should be documented and should exclude recycled or rendered materials in order to reduce the risk of prion-associated diseases.

To provide a high degree of assurance of product safety, animal donors and tissues should be screened at several stages throughout the process to rule out the presence of microbial agents. These control tests should utilize assays that are sufficiently sensitive and specific to detect bacteria, mycoplasma, fungi, or viruses of interest. Donor animals should be screened for relevant diseases before tissue procurement. Post-tissue-retrieval necropsies, sentinel animal programs, and archival storage of donor organs, tissues, blood, and other specimens also ensure the safety of animal tissue for use in cellular therapeutic applications.

In general, similar aseptic procurement issues apply to animal and human tissues. The tissue should be obtained under environmental conditions and controls that provide a high degree of assurance of aseptic recovery. Specifically designed procurement facilities, usually closely associated with the animal holding facility, should be employed. Recommended design features and attributes of the animal tissue procurement facility should include the following: (1) staging of events such as shaving, sedation, and operating room preparation in separate rooms with appropriate environmental controls; (2) high-efficiency particulate air (HEPA) filtration; (3) adjacent but separate facilities for further tissue processing; and (4) dedicated areas for carcass removal. Issues relating to personnel training, bowel manipulation and puncture, and disinfection apply to the surgical procurement of both human and animal tissues (see *Human Cells, Tissues, or Cellular or Tissue-Based Products,* above). When researchers establish animal cell lines for use as feeder layer cells, cell banks should be created, tested, and characterized as described in the next section.

CELL BANK SYSTEM

A cell bank is a collection of cells obtained from pooled cells or derived from a single cell clone or donor tissue that is stored in bags or vials under defined conditions that maintain genotypic and phenotypic stability. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although alternative approaches are possible. The MCB is produced in accordance with cGMP and preferably is obtained from a qualified source (one that is free from adventitious agents) with known and documented history. Human cells and tissues should be obtained by means of a licensed tissue acquisition vendor with a donor qualification program in accordance to 21 CFR 1271. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to production batch consistency because the starting cell material is always the same. However, it may not be possible or feasible to create a cell bank, so appropriately tested and qualified primary cells may be used in lieu of creation of cell banks. The MCB and WCB should be minimally tested for identity, sterility, purity, viability, and the presence of viruses and mycoplasma.

CELL BANK QUALIFICATION

Cell bank safety testing and characterization are important steps toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. ICH Q5A, Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin, gives specific recommendations for testing cell banks for viral agents. While this guide-

line is not specifically intended to cover cell or tissue-based products, the same tests are generally applicable. Additional virus testing may be needed depending on the prevalence of viral diseases endemic in the donor population. Testing to qualify the MCB is performed once and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB should be prospectively established. It is important to document the MCB history, the methods and reagents used to produce the bank, and the storage conditions. All the ancillary materials required for production of the banks, such as media, sera, cytokines, growth factors, and enzymes, should also be qualified, documented, and appropriately tested.

SAFETY TESTING OF MCB AND WCB

Master Cell Bank—Safety testing to qualify the MCB includes testing to demonstrate freedom from adventitious agents and endogenous viruses. The testing for adventitious agents should include tests for bacteria, fungi, mycoplasma, and viruses. Freedom from adventitious viruses should be demonstrated using both in vitro and in vivo test systems and appropriate species-specific tests.

Working Cell Bank—Safety testing of the WCB is less extensive and generally focuses on the potential for introduction of adventitious viruses or activation of latent virus during the additional culture required to create the WCB. End-of-production (EOP) safety testing should also be performed to ensure that the cells can be expanded a known maximum number of generations while still producing an acceptable product. For information about which types of adventitious virus testing should be performed on the MCB, WCB, and EOP cells, consult *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* 〈1050〉.

CHARACTERIZATION OF MCB AND WCB

Characterization of the MCB and WCB includes identity testing to establish species origin, e.g., isoenzyme analyses to confirm the human origin of the cells. However, cell bank characterization should encompass additional assessments such as the following:

- Growth kinetics and population doubling time
- Morphological assessment
- Percent confluence at passage
- Cell counts
- Viability (pre- and postcryopreservation)
- Phenotypic expression of desired and undesired cell types (pre- and postcryopreservation)
- Monitoring of unique biochemical markers (pre- and postcryopreservation)
- Assessments of functional activity (pre- and postcryopreservation)
- Gene and protein expression analysis (pre- and postcryopreservation)
- Expression of immune histocompatibility antigens (HLA/M HC)
- Molecular fingerprinting
- Chromosomal stability

Biocompatible Scaffold Materials

Most natural or synthetic scaffold materials are regulated as medical devices, although scaffolds derived from human tissues such as dermis are regulated as HCT/Ps. When possible, use scaffolds that have previously been approved for other clinical uses because these materials should have already undergone extensive safety and quality testing. For applications in cell or tissue-based products, the scaffold material should allow cells to attach, proliferate, and migrate, and high porosity is often desired to facilitate cell seeding within the material. The scaffold must provide adequate diffusion of nutrients for cell health and release of cell-excreted products. The material must have adequate mechanical strength and must be amenable to manipulation, chemical modification, and manufacture. The scaffold material should be biocompatible, relatively inert, and immunologically benign.

Scaffolds can generally be classified as hard or soft. Hard scaffolds are used in applications where a specific shape is required, such as forming a blood vessel or a bladder. Soft scaffolds are used in applications where the product needs to conform flexibly to an existing shape in the body.

Scaffold materials can be synthetic or natural polymers, biodegradable or permanent. Biodegradation allows the scaffold to be resorbed or removed from the body without manipulation. The scaffold degradation rate must coincide with the rate of formation or regeneration of the tissue. The natural scaffold structure must replace the degrading scaffold in such a way that it maintains the structural integrity of the tissue or organ being regenerated. For example, a newly formed blood vessel must withstand both the internal blood pressure as well as external mechanical forces.

The most commonly used synthetic biodegradable polymer is polyglycolic acid (PGA). Polylactic acid (PLA) is also widely used, sometimes in combination with PGA. These polymers degrade within the body, are readily removed before degradation,

and have a long history of use in suture materials. Polycaprolactum (PCL), which exhibits a slower rate of degradation than PLA or PGA, is used in applications that require a long presence in the body.

Extracellular matrix (ECM) and its derivatives are natural materials used for scaffolds in the manufacture of cell–biomaterial combination products. Proteins such as collagen or fibrin and polysaccharides such as chitosan or glycosaminoglycans (GAGs) have also been used in growing cells to make combination products. Collagen is by far the most popular substrate for cells and has been molded into scaffolds for a variety of products, mainly in tissue-engineered skin applications. Cross-linking agents such as glutaraldehyde and water-soluble carbodiimides have been used to enhance the strength of natural scaffolds. Depending on the source of the material, natural scaffolds can be immunogenic.

When cells must proliferate after seeding, the scaffold and the supporting culture system must allow the exchange of nutrients and waste products. A thick, impermeable matrix will lead to regions of necrotic tissue. Many tissue devices are designed so that they can eventually be removed from the patient.

The safety and biocompatibility of the scaffold and product-contact materials must be established. A full battery of tests recommended by *Biological Reactivity Tests, In Vitro* ⟨87⟩, *Biological Reactivity Tests, In Vivo* ⟨88⟩, ISO 10993, or FDA Blue Book G95-1 should be performed. Process residuals and degradation products from the preparation of the scaffold should be quantified and limits should be established. The stability and storage conditions of scaffold materials should be established.

Qualification of Ancillary Materials

Ancillary products include a wide variety of raw materials and components used in manufacturing. They may include relatively simple materials or complex substances such as culture media, buffers, growth factors, cytokines, cultivation and processing components, monoclonal antibodies, and cell-separation devices.

Ancillary materials are not intended to be present in the final therapeutic product. Defined media formulations typically include components such as albumin and transferrin that are purified from animal or human sources. The purification, processing, and extensive testing of such components further minimize—but do not eliminate—the risk of viral or microbial contamination. Residual amounts of ancillary materials used in the manufacturing process, including recombinant proteins or other defined media components, may be potentially antigenic so their removal from the final product should be assessed, and appropriate limits should be established when necessary.

Known risks are associated with the use of ancillary materials in the production of cell-therapy products. The quality of ancillary materials used in the production of a cellular therapy product can affect the safety, potency, and purity of the product. Ideally, each ancillary material employed in the manufacture of a cellular or tissue-therapy product should be produced under conditions that are in compliance with cGMP. However, complex or unique substances essential for process control or production may not be available from suppliers that produce them in compliance with cGMP. In these situations, the cellular or tissue-therapy product manufacturer should develop a scientifically sound strategy for qualifying the raw material. A qualification program for ancillary materials used in cell and tissue therapy manufacturing should address each of the following areas: (1) identification and selection, (2) suitability for use in manufacturing, (3) characterization and acceptance criteria, (4) vendor qualification, and (5) quality assurance. Lot history files should be constructed for each ancillary material.

Conformance to established specifications should be compared to the data supplied on the Certificate of Analysis. Traceability is essential, and lot numbers for each ancillary material used should be noted in the productions records of the cell-based product. USP general information chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩ should be consulted for specific information about implementing an appropriate qualification program for these materials. Other USP chapters provide considerations about the qualification of specific ancillary materials (e.g., *Bovine Serum* ⟨1024⟩, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩, and *Growth Factors and Cytokines Used in Cell Manufacturing* ⟨92⟩).

Qualification of Excipients

During the final steps in the manufacturing process, excipients or substances that increase the stability of the therapeutic cells may be included. Examples of excipients include culture media, USP saline or other electrolyte solutions approved for injection, exogenous proteins such as human serum albumin, or cryoprotectants such as dimethyl sulfoxide (DMSO). Excipients are not intended to exert a direct therapeutic effect upon the patient; rather they are intended to contribute to maintenance of the quality attributes of the final cellular product. Because excipients will be administered to the patient along with the cells, particular attention must be paid to their qualification. In general, excipients that are already FDA approved for human use should be used whenever possible. If nonapproved excipients are used, a complete safety assessment should be done. For novel excipients such as cryopreservation solutions, appropriately designed preclinical safety studies may be needed.

MANUFACTURING OF CELL OR TISSUE-BASED PRODUCTS

Introduction

The manufacturing of cell or tissue-based products requires a number of operations and manipulations by individuals who are well trained in aseptic processing techniques. The technical competence of the personnel is particularly crucial to product

safety and efficacy. Autologous products present more challenges for cell and tissue processing because lot segregation, line clearance, and operational processes must be developed to decrease the chance of mix-up of patient-specific lots (see *Facility Design and Operation Considerations*, below).

Cell Isolation and Selection

The general principles for processing human or animal tissues following aseptic procurement are independent of the cell or tissue source. The manufacture of cell products may occur at a cell manufacturing facility located in close proximity to the clinical site or at a distant central cell-manufacturing facility. The source cellular or tissue material should be packaged in sterile, leak-proof containers and transported from the procurement area to the processing area under controlled conditions that maintain cell viability. The fluid medium in which the specimens are bathed during transportation should be optimized to maintain cell and tissue viability. This transport medium can be supplemented with antibiotics. The antibiotic levels in process buffers should be decreased and eventually eliminated during subsequent processing steps so that antibiotics are not present in the final cellular product. In the case of blood products or tissues containing substantial amounts of blood, the transport media or buffered electrolyte solution should contain an anticoagulant.

ISOLATION

Solid organs or tissues are usually dissected to expose a desired region. This material may be used as is for transplantation, or it may be further processed. If multicellular organoids (for instance, islets of Langerhans) or single-cell suspensions are desired, the tissue may be subjected to mechanical or enzymatic disaggregation. Physical disaggregation may be accomplished by the use of instruments that homogenize the material by imparting high shear forces or breaking the tissue into smaller pieces. Alternatively, the material can be pressed or passed through screens of defined mesh sizes.

Enzymatic digestion of the extracellular connective tissue is another common method for dissociating solid tissue. Various enzymes are used to accomplish this, including collagenase, dispase, trypsin, elastase, hyaluronidase, papain, and chymotrypsin. Enzymes with nuclease activity, such as deoxyribonuclease, may be added to digest nucleic acids released from damaged cells, preventing excessive cell clumping. At the end of the incubation process, the cell suspension may be subjected to a mild pumping action to further break up multicellular clusters into those of desired size or composition. Enzymatic and physical disaggregation methods are often combined to achieve the desired result.

Because blood and bone marrow cells are inherently suspensions, mechanical manipulation is usually limited to plasma and aggregate removal, which is accomplished by centrifugation and filtration.

Cell and tissue isolation activities involving open manipulation steps should be carried out in an ISO 5 (class 100) biological safety cabinet. The environment surrounding the biological safety cabinet should be suitable to maintain aseptic processing operations. For minimally manipulated HCT/Ps in closed systems, these environments may be controlled but unclassified. However, for cell and tissue-based therapies that are manipulated and manufactured under cGMPs, the environment surrounding the biological safety cabinet should be controlled and classified, usually as an ISO 7 (class 10,000) clean room. Precautions should be taken to segregate patient-specific tissue and cell isolates.

SELECTION

Cell suspensions often consist of a mixture of cell types that may require further processing in order to isolate a cell population of interest or to decrease the level of an undesirable cell type such as potentially contaminating tumor cells. Various cell isolation and separation techniques provide high yields of pure cell populations.

Cell populations can be selectively enriched by varying the force and duration of centrifugation. Separation can also be achieved by isopycnic centrifugation in which the cell suspension is centrifuged in a gradient medium that encompasses all of the densities of cells in the sample. Specifically designed continuous-flow elutriation centrifuges separate cell populations by subjecting a cell suspension to opposite centrifugal and fluid stream forces in a special chamber within the centrifuge rotor mechanism. Cell populations separate within the rotor on the basis of their various sizes and densities, and they are selectively eluted out of the rotor chamber by increasing the fluid stream force. Finally, other methods involve the addition of high-density agents such as hydroxyethyl starch to the cell suspension. Concentration and separation procedures such as these frequently result in cell loss because of clumping and aggregation.

Cell separation can also be achieved by applying techniques that take advantage of unique cytological or biochemical characteristics of different cell populations. Soybean agglutinin aggregates cells that bear a particular carbohydrate moiety expressed on mature blood cells, but not stem cells, allowing enrichment of the stem cells. Lymphocytes possess the CD2 antigen that acts as a receptor for sheep red blood cells. When sheep red blood cells are added to the cell mixture, the lymphocytes form rosettes around the sheep red blood cells and are then separated via differential centrifugation. Some applications take advantage of the ability of certain cell populations to adhere to the surface of specific solid substrates such as tissue-culture plastic, collagen-coated materials, and natural and synthetic polymeric scaffolds. The specifically bound cell type is selectively recovered onto the surface and removed from the initial cell suspension.

General Chapters

Monoclonal antibodies directed against specific cell-surface proteins can be used for both positive and negative cell selection. For example, a monoclonal antibody-bound cell population can be removed from the cell suspension after exposure to magnetic particles coated with anti-monoclonal antibody. The magnetic particles and their bound cells are removed from the cell suspension magnetically. Unlabeled cell suspensions can be poured over or incubated on surfaces such as plastic flasks or microspheres coated with monoclonal antibodies as a means of isolating particular cell populations. In addition, a fluorescence-activated cell sorter (FACS) can be used to separate different cell types by binding antibodies tagged with fluorescent markers to a particular cell type.

Other techniques enrich cell populations by destroying unwanted cells. For example, certain cell-bound monoclonal antibodies are able to fix and activate exogenously added complement, resulting in cell lysis. Some procedures use cytotoxic agents or mitotic inhibitors to selectively kill unwanted cells. These methods typically target cell subpopulations with high growth rates, such as tumor cells. Finally, an antibody can be conjugated to a toxic moiety, such as ricin, allowing delivery of the cytotoxic agent to the targeted cell population. Most of these procedures require several washing steps to ensure the removal of the dead cells, cell fragments, and cytotoxic agents from the final cell product.

Cell Ex Vivo Expansion and Differentiation

EX VIVO EXPANSION

A key issue for manufacturers of cell and tissue-based products is the ability to produce and deliver a therapeutically relevant dose of the required cell population to the patient. Depending on the application, the product may be a pure, homogeneous cell type, or it may be a mixture of different functional cell types. Many target cell populations are present at a low level or low purity in complex primary source tissues. In such cases, production of a therapeutic dose may be achieved only by specific enrichment and ex vivo expansion of the required cells.

Ex vivo expansion of cells may occur in suspension culture (e.g., T cells or hematopoietic stem and progenitor cells), adherent culture (e.g., mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, neuronal stem cells, or dermal fibroblasts), or a mixture of both (e.g., bone marrow stroma expansion). Numerous technologies exist for cell culture. Cells can be propagated in tissue-culture flasks (T flasks), in roller bottles, on polymeric scaffolds, or in nonrigid, gas-permeable bags, usually inside incubator units controlled for temperature, humidity, and gas composition. Multilayered, high-capacity cell culture systems composed of tissue culture plastic, multibag systems, and bioreactors using microcarriers enable expansion, harvesting, and formulation to be carried out in a closed system. Traditional small-scale fermenter units can be used for expansion of cells in suspension culture. It is also possible to expand adherent cells in such units either by providing a surface for attachment (microcarriers, coated beads, or disks) or by adapting the cells to propagate in suspension culture. Some culture systems are specifically designed for the propagation of cells for therapeutic applications. These tend to be closed systems that use disposable bioreactor cartridges in automated processing units with direct control of temperature, gas composition, and media perfusion rate. In some cases automated software allows patient–donor tracking and documentation of culture conditions and manipulations. These features are useful in the design and implementation of QC product release testing programs and for the QA documentation of processing runs.

In adherent culture, the cells are usually harvested from the surface upon which they have expanded. Methods of release include physical agitation, enzymatic cleavage, and chelation of metal ions and competitive inhibition of adhesion or matrix molecules. As described above, consideration must be given to the source, safety, toxicology, and residual testing for any reagent used to release adherent cells during manufacturing. Some product-specific systems do not require the release of adherent cells. Cells are expanded on a biocompatible synthetic or natural scaffold that is then applied topically (for example, engineered skin substitutes), or the cells are grown inside or outside of fibers for ex vivo perfusion (for example, hepatocytes in hollow-fiber devices to treat liver disease).

In all cases standard cell culture parameters should be optimized for maximum process efficiency. Such parameters include composition of cellular source material, initial seeding density, media composition, rate of media exchange, temperature, gas composition, pH, and rate of delivery. Depending on the nature of the product, the potential effect of process parameters on the potency and function of the target cells should be defined.

Bioreactors—Specialized bioreactors and devices are required for manufacturing three-dimensional combination products. These bioreactors hold the biocompatible scaffold/matrices for the manufacture of the construct. Although the bioreactor can provide a closed system for construct manufacturing, it creates a challenge in providing access to the scaffold for seeding cells and sampling for product release testing while maintaining sterility. Bioreactors are often single-use devices that ensure that no cross-contamination occurs between products. Preferably the product will not be repackaged for transport and delivery. For example, bioreactors may also serve as the final container for product shipment.

Container–closure testing must be performed for all final container–closure systems. Compatibility for sterilization of the bioreactor and the scaffold should be verified, and the sterilization process must be validated for each product configuration. Leachables and extractables from product-contact materials such as bioreactors and packaging components should be quantified, and limits should be established.

In closed bioreactor systems it can be difficult to observe or sample cells. Measurement of metabolic parameters can provide a surrogate method that is amenable to validation with which to evaluate the rate of proliferation and predict when to harvest the cell product. The relationship of such parameters to the viability, potency, and function of the cell product should be well

defined. Postexpansion purification and enrichment of target cells by using methods such as those described above may be required.

DIFFERENTIATION

Some cell therapies require lineage or functional differentiation of the source cells. For example, hematopoietic stem cell expansion processes normally result in products that contain a mixture of multipotent stem cells, lineage-committed progenitor cells, and lineage-differentiated cells. The composition of these products can be manipulated by different combinations of growth factors and cytokines during the expansion process. The inverse is true for processes in which mature cells are *de-differentiated* to enable them to then be recommitted to a lineage pathway (for example, chondrocytes in cartilage repair). Specific examples of ex vivo manipulation are the production of antigen-specific T cells to target various specific disease indications or derivation of therapeutic cell types from embryonic stem cells. Before release for clinical use, the resulting differentiated target cells must be fully characterized. Assessing the potential for de-differentiation of multipotent cells that have undergone differentiation may be necessary to ensure the safety of the product. Where the cells have been expanded and subsequently differentiated, karyotype analysis or in vitro transformation assays may be performed to demonstrate the cells are acceptable for clinical use.

EX VIVO GENETIC MANIPULATION

Genetic modification of cells ex vivo is a common processing procedure that is used to alter the pattern of gene expression in a defined population. The introduction of integrating or nonintegrating genetic materials (DNA, RNA, siRNA) is performed in order to induce the expression of new genes and products or to inhibit endogenous gene expression. Ex vivo genetic modification in autologous transplantation settings involves the manipulation of a harvested or expanded cell population from a patient and subsequent readministration of the cells to the donor. In a typical allogeneic transplant setting, a stable, genetically modified cell population that has been characterized and banked is administered to a broad patient population. In order to control graft-versus-host disease in allogeneic bone marrow transplants, selected donor T cells have been treated with lethal genes such as thymidine kinase that make the cells susceptible to gancyclovir treatment after transplant. Examples of autologous genetically modified cell therapy products include the transduction of tumor cells with cytokine or other immunomodulatory genes, lymphocytes transduced with receptors for tumor antigens, and the introduction into harvested lymphocytes of an antiviral ribozyme vector as a strategy to treat human immunodeficiency virus infection. Allogeneic cell therapy product examples include genetically modified and irradiated tumor cell lines used as tumor vaccines and encapsulated cells transfected with a gene to express a neurotrophic factor for localized therapeutic protein delivery in the central nervous system.

Ex vivo genetically modified cells are considered gene therapy. Issues associated with gene therapy products are addressed in detail in *Gene Therapy Products* 〈1047〉, especially the production of the vector or genetic material used to accomplish gene transfer, analytical testing strategies, patient safety, and monitoring. The manufacturing, cell processing, and process control methodologies addressed above are applicable in the procedures used for genetic manipulation. Frequently cell populations that are genetically modified are isolated and expanded or selected before the introduction of the genetic material. Specialized equipment and processes for introduction of genetic material exist and must be validated and monitored. Issues associated with cell banking and stability apply to cell lines used in allogeneic cell therapy products that are established and cryopreserved in MCBs and WCBs. Finally, issues associated with analysis and administration of the genetically modified cell population are discussed later in this chapter.

Formulation of Cell and Tissue-Based Products

Approaches for formulating cell and tissue-based products depend largely on the planned storage time for the cells before delivery to the patient. For some cell-based products, the time between completion of manufacturing and delivery to the intended recipient can be measured on the order of hours to days. Other cell-based products may be cryopreserved in order to extend their shelf life. A different approach for formulating cell and tissue-based products may involve the addition of a natural or synthetic scaffold that can facilitate handling, protecting the cells from immunological responses, and creating a specific shape that contributes to the therapeutic effect. Considerations for formulating each of these types of cell and tissue-based products are discussed below.

NONCRYOPRESERVED CELL AND TISSUE-BASED PRODUCTS

Products consisting of suspensions of cells for delivery to patients within hours after the completion of manufacturing frequently are formulated in sterile, buffered solutions suitable for direct administration. For other noncryopreserved cell or tissue-based products extension of shelf life from hours to days may be possible by use of solutions that contain appropriate nutrients and antioxidants. In most cases these excipients are not intended for direct administration into patients. Consequently, the excipients may require removal before delivery to the patient (see *Clinical Site Preparation and Administration*). If an unapproved formulation buffer will be administered to patients, preclinical toxicology testing should be performed.

CRYOPRESERVED CELL AND TISSUE-BASED PRODUCTS

Most cell cryopreservation medium formulations are supplemented with 5% to 10% DMSO with or without hydroxyethyl starch (generally 6%) and a plasma protein such as 4% to 10% human serum albumin in a balanced salt solution. DMSO prevents dehydration by altering the increased concentration of nonpenetrating extracellular solutions during ice formation. The high molecular weight polymeric hydroxyethyl solution protects the cells from dehydration as water is incorporated into extracellular ice crystals. The use of protein often results in maximum recovery and viability of cells after thawing. Serum (5% to 90%) is sometimes used in place of specific proteins. Some cryopreservation formulations are completely free of protein.

The optimal concentration of cells for cryopreservation depends on the cell type and should be determined empirically, but it generally ranges from 10^6 to 10^7 cells per mL. The homogeneity and viability of the cell population being cryopreserved can also differ after thawing and should be carefully assessed. In situations where the final cell or tissue-based product is intended to be thawed and administered immediately, the presence of DMSO in the formulation buffer does subject the patient to an increased level of infusion-related toxicity, although this is related to the volume administered and the final concentration of the cryopreservative. Refer to section *Clinical Site Preparation and Administration* for additional considerations.

CELLS COMBINED WITH BIOCOMPATIBLE SCAFFOLDS

Many cell and tissue-therapy products are administered in combination with a biocompatible scaffold. For instance, wound healing or skin substitute products contain cells seeded on a scaffold. The biochemical and physical structure of the scaffold and the method for combining cells with the scaffold are specific to the product.

Cells can be loaded into a semipermeable membrane device for delivery. Usually the pore size of the membrane is large enough to allow the cell-secreted therapeutic factors to pass, but it is small enough to stop immunoglobulins and host cells from making contact with, destroying, or mounting an immune response to the therapeutic cells. The device can be a single hollow fiber or a semipermeable capsule with cells inside that secrete therapeutic compounds, or it can be part of a larger system of pumps and filters such as hollow-fiber modules with hepatocytes for the treatment of liver disease.

Cells can be seeded onto a three-dimensional scaffold and allowed to propagate and form a tissue-like structure. In the resulting product, the cells are oriented in a unique manner that is important for the intended use of the product (e.g., skin substitutes).

Cells can be encapsulated in a gel or cross-linkable polymer solution, and the resulting implantable structure can serve as a culture vessel, as a means to shield the cells from the host's immune system, or as a way to mold cells into a defined shape. Some of the polymers used include alginate, hyaluronic acid, collagen, chitin, or synthetic polymers. Encapsulated pancreatic β-islet cells have been implanted in patients to treat diabetes. To treat urinary incontinence, chondrocytes have been mixed with alginate to form a structure upon injection.

Cells can be adhered to scaffolds of defined shape that are then implanted. Some examples include osteogenic precursor cells on scaffolds of demineralized cadaveric human bone, ceramic hydroxyapatite, ceramic hydroxyapatite–tricalcium phosphate, or biodegradable glass, which can be used in the repair of bone defects.

ANALYTICAL METHODS

General Considerations

The complexity and scope of cell-based therapies are reflected in the wide range of analytical methods that are used to establish in-process controls and final product release criteria. Quality specifications for cell and tissue products should be chosen to confirm the product's quality, safety, and potency. Selected tests should be product specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and tissue products should follow the principles outlined in ICH Q6B *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products.*

Specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Manufacturers should develop specifications for each product developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to stability, safety, and efficacy assessments.

In-process controls and specifications for the product should be anchored by use of an appropriate reference standard. An autologous product may rely on a reference standard generated from processing cells or tissue from a healthy donor or from a source that supplies cells and tissues to research institutions. The reference standard ensures that the process, as measured by the release assays, does not change significantly over time, and it verifies that a test produces acceptable results, i.e. system suitability requirements are met. The reference standard is made from a lot that is produced under controlled conditions and passes all in-process and final release testing. In addition, this reference standard is subjected to an additional level of charac-

terization that includes tests not normally performed for product release. The reference standard need not be stored at the same dose, formulation, or temperature as the final product. However, the stability of this reference standard must be determined.

Alternatively, a working standard can be used. If so, in the test it should behave like the reference standard. Changing to a new reference standard should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted. One option for a reference standard for a cell product with a short shelf life or for an autologous or patient-specific application can be a bank of normal donor cells of the appropriate cell type. This cell bank can be used to ensure that the manufacturing process is capable of making a consistent product.

In-Process Controls

Manufacturing processes should have well-defined go–no go decision criteria that are established for key in-process manufacturing steps. In-process controls are the assays or tests that are performed to ensure that the in-process material is of sufficient quality and quantity to ensure manufacture of an acceptable final product. Examples of in-process controls include:

- Enumeration and viability
- Microbiological (sterility, endotoxin, mycoplasma)
- Expression of phenotypic or genotypic markers
- Verification of morphology against visual reference standards
- Production of a desired bioactive substance
- Determination of population doublings, passage number, age of culture
- Assays of potential process impurities
- Monitoring of culture system parameters (% CO_2, % relative humidity, pH, glucose, lactate, etc.)
- Functional tests such as colony forming units (CFU) and expression of cell-specific proteins.

A primary reason for establishing in-process control tests is to ensure that the correct product with specified quality and yield is obtained. A secondary reason for performing in-process tests is to gather process and product characterization data that can be useful in assessing the impact of process changes or excursions. Intermediate in-process material that fails to satisfy in-process control criteria should not be used for further manufacturing. This material can be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications, and the effect of reprocessing on other quality attributes such as stability must be defined before the material can undergo further manufacturing. If several sublots (e.g., cells harvested from different culture vessels) will be pooled for further processing, sublots that fail to satisfy specified criteria should not be included in the pool even if the pool containing these failed sublots would pass the in-process assay criteria.

During clinical process development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. Statistical process controls and critical parameters should be used to establish limits for process validations and manufacturing investigations. Statistical sampling tools should be used to ensure a valid sample size. In-process controls should be performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended, and actions should be taken to correct problems as they arise.

Final Product Release Specifications

Cell-based therapies regulated as biological products must comply with applicable sections of 21 CFR 211 and 21 CFR 610 to ensure their identity, purity, potency, microbiological safety, and other essential attributes, such as viability, are met.

Because terminal sterilization is not possible for a living cell-based product, essentially all cell-based products are required to meet acceptance criteria for product tests such as sterility, mycoplasma, and endotoxin—typically, negative or no growth demonstrates sterility and the absence of mycoplasma, and products must demonstrate <5 endotoxin units (EU) per kilogram of patient body weight. In the case of intrathecal injection, the specified endotoxin limit is more stringent ≤0.2 EU per kilogram of patient body weight. Adventitious virus testing is rarely performed on the final cell-based therapy product because the source cells or cell banks and ancillary materials of biological origin have been screened and tested for viral agents of concern before manufacturing.

For almost all other final product release criteria, such as those for identity, purity, and potency, the analytical procedures with methods and acceptance criteria are specific to the individual cell-based product. *Table 2* provides an overview of the expected final product release tests for cell-based therapies and lists examples of approaches that are used to satisfy the testing requirements.

Table 2. Overview of Final Product Release Testing

Release Test	Examples	Criteria
Sterility	USP ⟨71⟩	Negative
Mycoplasma	Direct and indirect culture method (FDA Points to Consider)	Negative; not detected

Table 2. Overview of Final Product Release Testing *(Continued)*

Release Test	Examples	Criteria
Endotoxin	USP 〈85〉	<5 EU/kg (<0.2 EU/kg intrathecal)
Identity	• Surface marker determination • Isoenzyme analysis • Genetic fingerprint • Morphology • Bioassay • Biochemical marker	Product specific
Purity	• Percentage of viable cells • Percentage of cells expressing specific marker(s) • Limits on undesired cell types • Limits on process contaminants (e.g., serum)	Product specific
Potency	• Viable cell number • Colony-formation assay • Change in expression of specific genes • Secretion of desired macromolecule • Induction of secondary effect (e.g., human leukocyte antigen (HLA)) • Evidence of metabolic activity • Evidence of cell function	Product specific
Dose	• Viable cell number • Enumeration of specific cell population • Total DNA • Total protein	Product specific
Others	• Appearance • Morphology • Size	Product specific

STERILITY

Cell-based products are required to comply with final product release testing requirements, including sterility. Sterility testing is also frequently performed in-process to establish microbial purity for cells that require extended culturing. Suitable sterility tests include the test described in 21 CFR 610.12 and USP general test chapter *Sterility Tests* 〈71〉. These culture-based test methods require 14 days and thus are suitable only for cell-based therapy products that have extended shelf lives (e.g., following cryopreservation). Many cell-based therapies have short shelf lives and must be delivered to patients before the 14-day test results are available. In such situations, FDA has identified an approach that will allow the administration of the cell-based product to patients in this setting [see *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*]:

- In-process sterility testing on a sample taken 48 to 72 hours before final harvest or after the last refeeding of the cultures
- A rapid microbial detection test such as a Gram stain or other procedure on the final formulated product
- Sterility testing compliant with 21 CFR 610.12 on the final formulated product

Under this alternative approach, the release criteria for sterility would be based on a negative result of the Gram stain and a no-growth result from the 48- to 72-hour in-process sterility test. In the event that the 14-day sterility test is determined to be positive after the product is administered to the subject, the manufacturer is required to report the sterility failure, results of investigation of the cause, and any corrective actions as an amendment to the IND within 30 calendar days after initial receipt of the positive culture test result.

Because of concerns regarding the sensitivity of a Gram stain and the inability to obtain full sterility results for 14 days after administration to the patient, there is widespread interest in the use of rapid microbiological methods as an alternative to the 14-day culture method. This is discussed under *Alternative Test Methodologies*.

MYCOPLASMA

Mycoplasma and ureaplasma are the smallest free-living microorganisms. Mycoplasma lacks a rigid cell wall and ranges in size from 0.2 to 0.3 µm. Mycoplasma can be observed as round or filamentous in cell culture using dark-field or phase-contrast microscopy. On solid agar, colonies of mycoplasma can range in diameter from approximately 15 to 300 µm, and the larger colonies are distinguished by a typical "fried egg" appearance.

Mycoplasma is ubiquitous and can be isolated from practically all mammals. Historically it has been one of the main problems in the contamination of tissue cultures. Mycoplasma tends to be fastidious and requires preformed nucleic acids supplied by media components. These components may be readily available in the cell culturing materials that are employed during manufacturing. Mycoplasma can arise from bovine or other animal-derived culture components, cell or tissue materials from asymptomatic patients, or possibly from operators who shed it during manufacturing.

Testing for mycoplasma is recommended for all raw materials derived from a human or animal source and is required as a lot-release assay for cell-based products. FDA has published a document (*Points to Consider in the Characterization of Cell Lines*

General Chapters

Used to Produce Biologicals) describing in detail the accepted methods for the cultivation and isolation of mycoplasma. Methods for mycoplasma detection are also described in USP general test chapter *Mycoplasma Tests* ⟨63⟩. Because the classical assay takes one month of testing to complete, alternative methods are being developed and validated for the rapid detection of mycoplasma. This is discussed under *Alternative Test Methodologies*.

ENDOTOXINS

Endotoxins exert a number of biological effects on the mammalian cell membrane and can affect secretion and cytokine production, can induce fever in recipients, or can serve as powerful mitogens. Because of the possibility of wide-ranging biologic effects of endotoxins on cell and tissue culture, raw materials and components used for the manufacture of cell-based products must be assessed for the presence of endotoxin as part of the raw materials qualification process. Control of endotoxin in the manufacture of cell therapy products is an essential element of any quality control program.

The presence of endotoxins in products administered to patients is a significant safety concern. USP general test chapter *Bacterial Endotoxins Test* ⟨85⟩ describes a number of different methods for measurement of endotoxins, all based on the *Limulus* amebocyte lysate assay. This assay can be validated for a wide range of biopharmaceutical products. An important feature of the assay with respect to cell therapy products is the ability to conduct the assay before release of products that have short shelf lives.

IDENTITY

Lot-release testing for cell-based products must include an identity test. This test serves unequivocally to identify the product. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous cell therapy product at a manufacturing facility where multiple patient products are manufactured by comparison with an allogeneic cell therapy that is the only product manufactured in a facility.

Identity tests for cell-based products must be relevant to the cell type and manipulations applied during processing. Differential surface markers are frequently used to ascertain product identity. Flow-cytometric immunoassay methods are the most common means of detecting and quantifying these markers. Identification and quantitation of particular cell subsets is accomplished by multiparameter analysis, usually of size and granularity and of one or more identity markers. Other examples of identity tests include isoenzyme analyses to confirm species of origin, which would be desirable if the product consists of xenogeneic cells. Cell morphology may be used to distinguish specific cell types. There is also an increasing trend to use genetic fingerprint technologies such as short tandem repeats to establish the identity of cell lines (e.g., human embryonic stem cells used to derive therapeutic cell types).

PURITY

Purity methods specifically quantify the intended active product components. Impurities are either product- or process-related residual contaminants that can be detected in the final product. The requirement to test for a particular impurity for product lot release will depend on the following: (1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity by process validation and (2) the toxicity potential or functional product impact associated with the impurity.

Examples of process-related impurities associated with cell therapy products include residual production-medium components (e.g., serum, antibiotics, or exogenous cytokines), ancillary materials used in downstream processing (e.g., nucleases or proteases), and leachables (e.g., plasticizers from tubing or culture plastic). Impurities may be bioactive (e.g., cytokines or hormones) or immunogenic (e.g., aggregates, degradation products, or animal-derived proteins). Impurities may have other deleterious effects, depending on the dose of the product.

Product-related impurities are specific to each product type. Examples include cell debris, presence of undifferentiated cells in a cell-based product that should contain specific types of differentiated therapeutic cells; unacceptable levels of nonviable cells; replication-competent cells in a cell product that should contain mitotically inactivated cells; or changes in the composition of functional cells following cryopreservation and thaw. Analytical methodologies to assess purity require quantitation or analytical separation of the intended product from its impurities. Emphasis should be placed on demonstrating the consistency of the product-impurity profile. It may be possible to validate the manufacturing process to the extent that specific lot-release testing for impurities can be limited.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

POTENCY

Potency is defined under 21 CFR 600.3(s) as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*, below). The relationship between product potency measurements during development and manufacturing to clinical safety and efficacy is key to their use in batch release. Potency may be assessed by in vitro or in vivo bioassays or a combination of the two. It is not uncommon for these assays to have coefficients of variation between 30% and 50%. These assays require a well-defined, representative reference material that can be used as a positive control for the assay. The positive control serves to qualify the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. High-precision assays are effective tools in monitoring product quality. Information about potency-assay variability should be incorporated in the stability study design and the proposed statistical approach to assignment of expiration date (see *Stability*, below).

The types of assays that can be used to establish the potency of a cell-based product vary widely and depend on its unique characteristics and its shelf life. For some cell-based products such as hematopoietic progenitor cells, assays for product potency have been correlated with clinical efficacy. In this case, a traditional colony-forming assay that quantifies committed progenitor cells such as colony-forming unit–granulocyte-macrophage (CFU-GM) has been correlated with clinical engraftment outcomes in some studies. For other cell-based products, in vivo animal models of disease have been used to establish product potency. If the cell-based product releases a bioactive macromolecule, a potency assay could be based on units of activity released. For example, the production of insulin in response to changes in glucose levels could be the basis of a potency assay for cells that are intended to treat diabetes.

Patient-specific products such as autologous immunotherapies present a challenge in demonstrating therapeutic activity in an in vitro or in vivo assay system. Novel approaches to measuring potency, such as the correlation of clinical outcome to other characterization tests such as identity tests, may be appropriate and should be discussed with regulatory authorities early in development. For example, the ability to determine specific cell-surface identity markers by employing flow cytometry techniques or vital stains may be an acceptable measurement of potency if properly validated and correlated with clinical outcome. FDA has issued guidance that discusses the possibility of using a matrix of biological and nonbiological assays, including both qualitative and quantitative assays, to establish product potency. Information in this guidance is particularly relevant for cell-based products that have a short shelf life or complex mechanisms of action or multiple biological activities (see *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*).

A validated potency assay is typically required before regulatory approval. During investigational clinical studies, regulators typically require that a well-defined assay or assays intended to establish product potency should be in place before the initiation of pivotal clinical trials. Early implementation of one or more candidate assays intended to establish product potency is strongly encouraged. Data from these candidate potency or functional assays can be particularly important when assessing proposed manufacturing changes, during technology transfer, and in determining product stability.

DOSE-DEFINING ASSAYS

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision. An assay that measures therapeutic activity of the product is referred to as a potency assay and it is designed to measure product function. This type of assay is different from a dose-defining assay. The design of the assay depends on the type of product. In the case of drugs, assays that measure the amount of active ingredient (dose) are referred to as strength assays. For these medicines, product dose can be defined as the concentration or amount of the final product administered to the patient, and it is typically measured as product mass. For cell-based products, attributes such as viable number of therapeutic cells are often used to define the dose of the product.

Cell therapy products may be dosed on the basis of enumeration of one or more cell populations. For products in the form of a homogeneous, single-cell suspension, viable cell number is the most frequently used assay. Such assays may include enumeration of all cells, total nucleated cells, or another subset of cells. Viability assays are usually based on a cell's ability to exclude a supravital dye, such as trypan blue. Results are expressed as the number of cells that exclude the dye and are therefore considered viable. Fluorescent compounds that bind to nuclear proteins and are excluded by viable cells may be incorporated into flow-cytometric methods for simultaneous determination of viability and cell-identity markers.

Cell counting may be performed rapidly by manual or automated methods. Manual cell counting by visual enumeration of cells in a hemacytometer chamber is a readily available technique with acceptable accuracy but a lower degree of precision than most automated methods. Typical instruments for automated cell counting provide reproducible enumeration of non-nucleated cells (e.g., erythrocytes and platelets) and nucleated cells and differential counting of the nucleated cells into mononuclear and polymorphonuclear leukocyte populations. Further discrimination of specific cell populations usually requires cell-surface phenotype analysis by flow cytometric or other methods (see *Identity* above). The proportion of a specific subpopulation of cells may be determined by FACS analysis or by flow cytometry. An example of a cell enumeration assay is the enumeration of CD34-positive (CD34+) hematopoietic progenitor cells.

For products that contain cells in a nonhomogeneous suspension, such as cells that are combined with a biomaterial (e.g., a scaffold), alternative measures have been used for cell enumeration, including total area of a cell sheet, wet weight, total pro-

tein, and total DNA. If such measures are used to determine product dose, then supplemental tests should be performed to establish relevance.

Considerations for Release Testing of Cell–Biomaterial Constructs

For some cell-based products such as cells combined with biomaterials to form combination products, it may not be feasible to directly test the cell–biomaterial construct. This is frequently the case when autologous cells are involved and the cell–biomaterial construct consists of a single unit and sampling of the construct is not feasible. In such cases, the individual components are tested before they are combined, and the final construct is not subjected to direct testing. Indirect measures such as sampling of the culture media can be employed to address regulatory requirements. The quality and stability of the formulated cell–biomaterial construct and relevance of indirect measures must be established by validation studies during product development.

Sampling Issues

As required by GMPs, product samples must be retained after release testing is completed. If rapid-release strategies are employed, manufacturers may need to retain additional samples so that product quality can be reassessed by alternative or traditional test methodologies if necessary.

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See *Stability Protocol Development* (below) for additional considerations. Samples from each lot should be retained in case there is a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling plan deserves special consideration. In such cases, process validation will assist in determining the appropriate statistically based sampling design.

Alternative Test Methodologies

As described under *Final Product Release Specifications* (above), cell-based therapies must undergo testing for sterility, mycoplasma, and endotoxin. Additional acceptance criteria for tests relating to identity, purity, potency, dose, and other relevant characteristics must be met before clinical use. With the exception of sterility, mycoplasma, and endotoxin, most of the test procedures and their underlying methods used to ensure that the final product meets release acceptance criteria are unique to the product and can be adapted to meet the specific characteristics and applications of the cell-based product. In general, test methods should be developed based on the best available science and should be suitable for use in a GMP manufacturing environment. The assays should be robust, reliable, and capable of being validated and should provide results before release for clinical use. *Validation of Compendial Procedures* 〈1225〉 provides basic considerations for methods validation.

For some cell-based therapies, the sample size and volume of material required for testing or the length of time necessary to obtain test results can consume significant amounts of the final product, or the time required for obtaining results may exceed the product's shelf life—or both. This creates problems with the available supply of product to treat patients and in other situations precludes the possibility of obtaining results before administration to patients. This is a particular problem for the compendial sterility test as well as the FDA-recommended broth-agar culture method for mycoplasma. Consequently, both industry and regulatory authorities have shown considerable interest in facilitating the development of alternative test methods for both sterility and mycoplasma.

FDA regulations for biological products specifically address the use of equivalent methods provided they ensure that the safety, purity, potency, and effectiveness of the biological product is equal to or greater than the assurances provided by the specified method (21 CFR 610.9). Some of the available alternative test methods for sterility and mycoplasma are describe below.

The range of available technologies is broad and continues to be developed by assay designers for use in the cell therapy industry. Attributes that should be included in any review of proposed technology include accuracy for the intended purpose, speed in productivity, cost, acceptability by the scientific community and regulatory agencies, simplicity of operation, training requirements and reagents, the reputation of the vendor, technical services provided by the vendor, and, finally, utility and space requirements.

Validation of these test methods and demonstration of equivalence as described in 21 CFR 610.9 are required at the time of biologics license application (BLA) or a premarket approval (PMA) submission.

STERILITY

Detection platforms for alternative microbiological methods have been generally based on growth, viability, artifacts, or nucleic acids. Growth-based technologies use either biochemical or physiological measures that reflect the growth of microorganisms. Test samples are transferred to traditional or enhanced media formulations that encourage microbial proliferation, and microbial growth is detected chemically or spectrophotometrically. The primary advantage of these systems is the automated nature of the test results and recovery of microorganisms for failure investigations and other microbial characterization

methodologies. FDA has published guidance for validation of growth-based rapid microbiological methods (*Guidance for Industry: Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene Therapy Products*, CBER, 2008). Principles of validation of alternative microbiological methods are also described in USP chapter *Validation of Alternative Microbiological Methods* 〈1223〉.

Viability-based technologies do not require cells to grow. These technologies are based on detecting the presence of individual living contaminants by vital dyes, stains, or cell-surface markers. Cells labeled by a specific fluorochrome metabolic substrate are collected on a membrane for detection.

Artifact-based technologies analyze cellular components or molecular probes that are designed specifically for a particular microbial species. For example, individual species can be characterized by unique patterns of fatty acid composition after samples of whole cells have been saponified to induce the formation of methyl esters. Other technologies use time-of-flight mass spectrometry.

Nucleic acid technologies (NAT) are based on polymerase chain reaction (PCR) DNA amplification, 16s or 23s rRNA typing, and gene sequencing. Some technologies identify microorganisms by sequencing a portion of the chromosome of an unknown organism and comparing the sequence of 16s rRNA to a database. This technology is capable of identifying fungi, mycoplasma, and bacteria, including slow growers and nonfermenters. For more details on PCR-based methods, see the USP chapter *Nucleic Acid-Based Techniques—Amplification* 〈1127〉.

MYCOPLASMA

Compendial testing methodologies for mycoplasma are growth based in agar and broth cultures and require at least 28 days to monitor appropriately the presence of mycoplasma contamination. Because of this limitation, a number of rapid mycoplasma testing technologies have been developed based on nucleic acid amplification techniques such as PCR, as well as nonamplified nucleic acid hybridization assays, ELISA, and enzyme-based assays.

QUALITY SYSTEMS

Quality systems weave together the various aspects of manufacturing. Quality control (QC) and quality assurance (QA) programs should exert control over the manufacturing facilities, the manufacturing process, the validation efforts, and all testing of the raw materials, in-process material, bulk product, and final formulated product. Training and certification programs are central to maintaining a technically competent manufacturing staff. A documentation program should be implemented to support all manufacturing, training, validation, and quality operations. Changes to processes and procedures should follow a formal program based on well-established change control principles.

When allogeneic human cells or tissues are used as the source material for the manufacturing of cell or tissue-based product, cell or tissue donors should undergo appropriate screening and testing (see *Donor Eligibility*, above). In all cases the source human cells and tissue must be handled in accordance with GTP as described in 21 CFR 1271.

In addition to GTPs, cGMP as outlined by FDA in 21 CFR 210, 211, 600s (especially 21 CFR 610), and 820 apply to the manufacturing of cell and tissue-based products that are subject to premarket approval. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of cGMP. GMPs apply throughout the clinical development to both the manufacturing process and facility. The extent of control increases as clinical development progresses, and full cGMP compliance is expected by the time Phase III clinical trial(s) begin.

Data obtained from in-process and final product release testing should be monitored. Results that are out of specification (OOS), or even those that are out of trend, must be investigated before disposition of the material. FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* (October 2006) provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as an analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that results of tests of the product do not fall with specified acceptance criteria, the lot should be rejected. In some situations, especially with autologous or allogeneic patient-specific product, a product that does not meet all specifications or that has only incomplete test results may have to be administered to a patient as a life-saving measure. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results to all parties, including regulatory authorities.

As discussed earlier an effective risk-management approach at the earliest stages of product development can ensure the highest quality of a cell-based product by providing a proactive measure to identify and mitigate potential quality issues. The probability for failure in cell therapy products can arise from a number of sources including personnel errors, aseptic processing failures, equipment failures, facilities and utilities failures, cleaning, disinfection, and component and raw material failures. A proactive understanding of risk can lead to improved decision making if a quality problem arises. Effective risk management can facilitate better and more informed decisions and may provide regulators with greater assurance of a developer's ability to deal with potential risks. Such assurance can affect the extent and level of direct regulatory oversight. Quality risk management can be integrated into key parts of the quality system such as change management, Corrective and Preventive Action (CAPA), GMPs, validation, etc., and can be used to establish meaningful specifications and Critical Process Parameters (CPPs) to ensure that the quality attributes are met.

Risk analysis is qualitative in nature. It can be achieved by using experience and process knowledge to define risk categories that can form the basis of a system of risk assessment and mitigation after the identification of manufacturing errors. For example, it is common practice to develop nonconformance or deviation risk assessment categories that can be incorporated into a nonconformance or failure investigation procedure. The categories are particularly useful if the risk assessment must be expedited to facilitate a CAPA. As an example, Risk Levels 1 through 4 are defined below and can be adopted as one means of conducting a preliminary risk assessment:

Risk Level 1: *Technicality*—Poses no risk to the patient and does not impact the safety and effectiveness of the product. Example: A missing signature on a batch record.

Risk Level 2: *Alert*—May pose a safety risk to the patient or may have a potential impact on the safety and efficacy of the product. Compliance must be re-established with appropriate justification to proceed after submission to QA for review and approval. Example: Digestion time for biopsy processing falls outside a defined range.

Risk Level 3: *Do not ship/reject lot*—May pose a safety risk to the patient or impair the efficacy of the product even after corrective action. Shipment is not permitted. Example: Cultures fail to demonstrate adequate cell growth.

Risk Level 4: *Post-distribution Event*—May pose a safety risk to the patient or a potential impact on the safety, potency, or purity of the product. The safety signal is identified after product distribution. Example: Failed sterility test occurred after distribution of product.

FACILITY DESIGN AND OPERATION CONSIDERATIONS

Manufacturing facilities for cell and tissue therapy products must be carefully designed to maintain GMP aseptic processing operations while also accommodating any unique aspects of the product. Incoming cells or tissue can have bioburden and other contaminants and may need to be received and processed in a segregated area under quarantine to avoid compromising the main facility. Also, tissue processing to obtain cells of interest may require specialized equipment and processes that should be considered during the facility design and subsequent operations. For manufacture of combination products involving biocompatible scaffolds, the facility may need to be capable of handling operations that involve chemical processing, handling, and disposal. This may place constraints on the design of the facility, especially air-handling systems in clean room environments.

Although the primary emphasis in manufacturing a cell or tissue-based product is protection of the product from inadvertent contamination, risk to the manufacturing operator must be assessed and minimized by appropriate training for handling blood-borne pathogens and the use of equipment/protective clothing. Protection of the operator and aseptic processing are complementary and include the use of certified biological safety cabinets and aseptic protective clothing consisting of gowns, gloves, sleeves, surgical masks, eye protection, and head coverings. Human tissue should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery.

The degree of control required for cell and tissue processing operations depends on a number of factors, including the complexity of the aseptic manufacturing process, the primary site of manufacturing, and the final product shelf life. Manufacturing processes that involve open manipulation of cells, even in a biological safety cabinet, are at greater risk of contamination than are processes done in closed bioreactors or bag systems that use sterile connections and tube-sealing devices. Typically, ISO 7 (class 10,000) clean rooms and ISO 5 (class 100) biological safety cabinets are essential components for cell therapy manufacturing processes, especially those that involve open manipulations.

The controlled environment of a carefully designed, constructed, validated, and maintained clean room can minimize the risks of environmental contamination during aseptic processing and decrease the possibility of cross-contamination of patient-specific products. The differential pressures between classified manufacturing should comply with the September 2004 guidance document, *Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*. The facility and processing areas should be monitored for air quality in a manner that provides a high level of process asepsis. For guidance in this area, see *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩.

Facility cleaning, component and product segregation, and sanitization procedures must be in place to avoid microbial contamination and cross-contamination between lots produced in the facility.

Cell or tissue products based on autologous cells add another level of complexity to the manufacturing facility design and operation. For autologous products one product lot is made for each person, and complete segregation during manufacturing is needed. Unlike facility designs for allogeneic products, which are based on volume scale-up to achieve maximum manufacturing efficiency, facilities for autologous products require unit scale-up (scale out), which must be considered in the design and operation of the facility. Automation can be used effectively to manage repetitive manual manipulation of cells. The initial scale of operation may not justify an upfront capital investment in automation but should be considered as manufacturing operations increase in size.

Product segregation in the facility is another key consideration for design and operation. Labeling and QA oversight are traditionally used for tracking and segregation. Techniques such as bar-coding and radio-frequency (RF) tags can be used for product tracking and segregation. For guidance in this area see 21 CFR 211.42, 211.113, 1271, and FDA's September, 2004 *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*.

Manufacturing equipment should be robust, should provide consistent product, and should allow periodic calibration and preventive maintenance. Qualification is necessary for equipment from which critical process parameters or measurements are derived. In most cases, this includes the software that controls the equipment or system's operation. Critical equipment such

as incubators and freezers need to be fitted with alarm systems that can remotely signal failure. Additionally, critical equipment should be connected to an emergency back-up generator, and the generator should be tested periodically to verify its operational state.

CONSIDERATIONS FOR VALIDATION AND QUALIFICATION

The principles of validation recommended by ICH and FDA guidance documents and USP chapters ⟨1225⟩ and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩ apply to cell or tissue-based products. Biological variation in the source cells and tissues used to create most cell or tissue-based therapies may affect validation efforts.

Validation activities should include risk assessments, training and personnel qualification, equipment and facilities qualifications, analytical methods validation, aseptic processing, the manufacturing process, and cleaning.

Process validation should take into account safety, consistency (process and step yields, clearance of impurities), robustness (operator-to-operator, day-to-day), and final product quality (identity, purity, potency). Analytical methods used to assess the process should be validated or well qualified.

Process validation for patient-specific products such as autologous cell and tissue products presents some unique issues. First, the starting materials for patient-specific products typically arise from patient or matched donor materials such as biopsy material, apheresis products, scrap tissues from surgical procedures, and cadaver organs that do not qualify for transplantation. The process may require manufacturers to accept a range in the quality and quantity of starting material and yet still produce a final product that satisfies release testing. Second, manual processing of cells and tissues exhibit a degree of inherent variability. Processing steps should be developed that successfully and consistently result in appropriate process components and final product, even if the process relies on nonstandard or variable tissue materials. Process validation should take this variability into consideration and should ensure that critical manufacturing and testing endpoints consistently meet specifications.

Aseptic process validations should be performed using microbiological media to show that the manufacturing staff can execute the procedures and produce a product free of microbial contamination. Procedures intended to maintain segregation during manufacturing should be challenged to verify that there is minimal opportunity for cross-contamination or mix ups among different patient product lots.

Depending on the variability in the source cells or tissues and the complexity of the manufacturing process, it may be necessary to manufacture more than three qualification lots to verify the consistency and the robustness of the manufacturing process. Not every manufacturing effort will be successful for autologous and patient-specific therapies. However, the success rate should be established and tracked to enable manufacturers to discover any decrease in that rate and to take actions to correct the problem. Well-characterized banked primary cells may be used in the validation of the process if the donors have a range of profiles expected for the patient population. Trending of a number of statistically acceptable product administrations can also be appropriate.

Equipment and facility cleaning validations should be performed to demonstrate the efficacy of cleaning agents on standard microbial and fungal contaminants as well as environmental contaminants isolated from the manufacturing facility. Measurement of residual cleaning agents should be addressed in equipment cleaning validations. Equipment, facilities, and electronic monitoring and control systems such as building monitoring and inventory control systems should be validated.

Analytical and manufacturing equipment and methods should be validated following the principles described in ⟨1225⟩ in addition to guidance documents issued by ICH (see Q2 R1). Training plans should be established, and personnel qualifications should be performed. Tissue transport and product shipping validations should be performed.

CLINICAL SITE PREPARATION AND ADMINISTRATION

General Considerations

Before administration of some cell or tissue-based products, one or more product modifications or preparative steps may be required. These modifications or steps are frequently performed close to the time of administration, and, therefore, they are not under the control of the original manufacturer. The nature of these modifications is largely dictated by characteristics of the product.

Preparative steps may include thawing, washing, or filtration to remove unwanted cells or substances accumulated during storage, transfer to an infusible solution, or formulation with a vehicle or structural material such as a scaffold. In addition, patient considerations, such as the need to dose or modify the product according to the patient's anatomical structure, weight, or blood volume may influence these steps.

At the clinical site, additional procedures and process controls must be established for all product storage intervals, transport steps, and modifications, starting with a clear definition of critical control points. Operational requirements include designation of a physical space suitable for aseptic handling, such as an ISO 5 (class 100) biological safety cabinet, trained personnel, detailed standard operating procedures, and quality oversight. The unique and irreplaceable nature of many cell or tissue-based products heightens the need for well-established procedures for clinical site preparation and administration.

Product Manipulations

Before administration of medical products that contain cells, on-site preparation may involve one or more manipulations. Typical manipulations include the following:

Change in Final Container—The manufactured product may have been stored or transported in one container and may require transfer to a different container for administration.

Change in Physical State or Temperature—A product may require thawing or warming.

Change in Solution or Suspension—A product may have to be dissolved, diluted, or suspended in a liquid.

Combination with a Biomaterial—Therapeutic cells may require combination with a scaffold material such as decellularized extracellular matrix sheets, gels, plugs, capsules, sponges, particles, or granules. In other cases, cells can be added to an existing medical device such as a hollow-fiber filtration unit before use.

Admixture or Compounding—For some cell products, mixing or compounding at the clinical site may be necessary.

Filtration or Washing—The presence of unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations, may require washing or filtration steps.

Sampling—Sampling of the final product before administration may be required to test the final formulation.

Clinical Site Facility Considerations

Facility requirements for performing on-site preparative steps or administration of cell therapy products depend on the products and the manipulations required. The most important determinant of facility features is the level of risk for microbial contamination associated with each step. See *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩ for guidance that relates the type of manipulation and levels of environmental control needed to ensure aseptic handling.

Thawing Cell-Based Products

Thawing is performed rapidly. If a small number of cells will be reinfused or transplanted, DMSO does not need to be removed from the suspension because most cell preparations can be concentrated adequately to keep the DMSO concentration within tolerable limits. DMSO use has two effects on cells after thawing: Cells may clump if damaged, and DMSO reduces cell viability in minutes. If the DMSO must be removed or cells must be concentrated for administration, the thawed cell suspension is generally serially diluted (to avoid osmotic shock) and resuspended in a protein-containing medium. Cell viability and potency may be monitored after thawing, but the information is frequently intended only to gather information rather than as a specification that must be met for clinical use of the cellular product.

Some cell therapy products require that the product be shipped fresh (i.e., not frozen). In certain situations cellular components are stored frozen but are thawed and applied to scaffolds at the manufacturing site just before shipment at ambient or refrigerator temperatures. Combination products composed of cells on a scaffold may require shipping at higher than refrigerated temperatures (i.e., room temperature) to avoid dislodging cells from the scaffold. Because the fresh product is metabolically active, the shipping container must be designed and validated to maintain the metabolic activity of the product in addition to the standard shipping validation testing. Metabolism can be slowed down by lowering the shipping temperature, but this requires reliable temperature control during shipment. Because shipping containers depend somewhat on the outside temperature, the shipping container must be validated to maintain strict temperature control in all weather conditions.

Additional Release Testing of Clinical Site-Manipulated Cell Products

Cell therapy products that undergo preparative steps or manipulations at clinical sites must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations determines whether additional release requirements or critical specifications must be added to those required immediately after initial manufacture.

Prerelease steps usually include the following:

- Physical inspection of the product, including product appearance (color, turbidity, particulates, or foreign matter), container integrity, temperature, and accuracy and convenience of labeling
- Review of process records and/or certificate of analysis
- For patient-specific products, verification of product labeling and records related to identity of the intended recipient

High-risk products (defined in ⟨797⟩) should undergo additional testing. For all high-risk products, assess the need for and, as appropriate, perform additional quality assays for the identity, potency, and purity of the active ingredients. For high-risk products in Category II, perform sterility and endotoxin testing.

Administration to Patients

Depending on the specific cell or tissue therapy application, patient-care staff may be required to take certain steps to prepare the patient. These steps help ensure that the product will provide the intended therapeutic outcome and help minimize the risk of adverse effects.

Determination of patient suitability for the therapy, including histocompatibility evaluation, typically occurs before the product is prepared. A patient's clinical status can change after tissue collection (because of fever, infection, recurrence or spread of tumors, or organ dysfunction), so the patient's general condition and suitability for therapy should be reviewed before product administration. This evaluation may include a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, relevant baseline physical or functional measurements, laboratory tests, or imaging studies may be obtained.

Depending on the route of administration, the patient may require preparation before treatment. For cellular therapies that require intravenous administration, patients with impaired peripheral circulation may require placement of a central venous catheter. When cells or tissues combined with structural materials are implanted into the patient, the site requires preparation. This may involve establishing surgical access to the site, removing degenerated or damaged tissue, trimming adjacent tissue to accommodate the implant, and excising tissue from a second site for anchorage or support for the implant. For instance, in the case of cell products for wound healing, the site for grafting must be free from infection and must have a well-prepared wound bed. For cells to repair cartilage defects, the site of damage needs to be prepared. Before direct therapeutic administration into an organ system (e.g., the bronchioalveolar system) or vascular network (e.g., coronary arteries), the patient may require surgical, endoscopic, or radiographically directed catheter access.

In all cases, adequate anesthesia and premedication must be carefully evaluated. For example, if DMSO will remain in a thawed, cryopreserved cellular product, the patient may be given an antihistamine before administration. Pre-administration evaluation must also include assessment of concurrent therapies that may interact with the cell or tissue-therapy product to modify its effects. Some therapies may be adjunctive to the cell or tissue therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects.

DELIVERY OF CELL-BASED THERAPY TO PATIENTS

Some cell or tissue therapy products are patient specific because they are manufactured from a selected autologous or allogeneic tissue source, cells, or tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, and at least two people should verify the identity of the patient and patient-specific product immediately before administration.

Cell and tissue therapy products can be administered by a variety of routes, including the common parenteral routes (intravenous, subcutaneous, intramuscular, and intra-arterial) and the respiratory or gastrointestinal tract. Other possibilities include direct application into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. Although parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities such as an aseptic operating theater or endoscopic suite. A variety of delivery systems such as catheters, syringes, and IV lines are frequently used to administer cells to patients. Before clinical use manufacturers should ensure that these medical device components are compatible with the cells and formulation solutions. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

POST-ADMINISTRATION MONITORING

Written policies and procedures for monitoring patient outcomes and for reporting and managing adverse events should be in place. Patient outcome assessments should include indicators that are likely to detect errors or problems related to the entire manufacturing process, and special attention should be given to manipulations, storage, or transportation after manufacturing. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients and a system for reporting and evaluating adverse effects that may identify potential product defects. Reporting includes information required for federal or state adverse-event monitoring programs.

STABILITY

General Considerations

The stability of cell or tissue products and the components used to create them will vary depending on the nature of the product, its intended clinical use, its specific attributes, and storage, packaging, and shipping conditions. For this reason, comprehensive guidelines covering a broad array of products are usually not possible. In all cases stability studies should be based

of scientifically sound principles and a comprehensive understanding of the final therapeutic product and its intended use. Manufacturers also should assess the stability of in-process hold steps, cell banks, critical raw materials, and reference standards. A well-designed and executed stability program provides a high degree of assurance that the product is stable during its specified shelf life.

Where feasible, stability testing should be carried out in accordance with the principles described in ICH guideline Q5C, presented in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* ⟨1049⟩. Stability data should also be collected for bulk and other in-process materials that are stored before final processing and filling.

Depending on the formulation used and storage conditions, shelf life can vary from hours to years. If a product has a short shelf life or if a product stabilizer must be removed, the final formulation may require preparation at the clinic just before administration. Instability is frequently observed as aggregation in cell products and as structural nonuniformity in tissue products. The stability of the final cell or tissue product must be established by validation studies during development.

For some cell and tissue products such as autologous or patient-specific cell products, final lots tend to have small volumes and perhaps shelf lives of only a few hours or days. In such cases, stability protocols should be based on materials from multiple donors. Because it is frequently difficult to obtain sufficient cells or tissues from autologous or patient-specific products, cells or tissues from several sources such as normal donors, research tissue repositories, cadaveric sources, or well-characterized banked primary cells can be used in stability studies to validate storage, shipping, and expiration dating. However, the results obtained with such "surrogate" cells or tissues must be interpreted cautiously and conservatively until data confirm that the actual autologous or patient-specific cells exhibit stability profiles that are similar to those of the surrogate cells or tissue.

For combination products that include cells and biomaterials, the stability of both components must be considered. When biodegradable scaffold materials are present, scaffold degradation should be considered in determining the stability and shelf life of the combination product.

Stability Protocol Development

Formal stability studies to support licensure and early-phase product stability information gathering should be detailed in a written plan that describes how stability data will be collected and analyzed to support the expiration date. Protocols should follow the format recommended in existing regulatory guidelines and should include the scope, storage conditions, and number of lots to be tested, test schedule, assays, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the reasonably expected challenges the product may encounter. For instance, if the final product formulation is performed at the clinical site, stability studies on this final formulation should establish the time frame and conditions under which the product can be held. For cryopreserved cells or tissues, stability should be established following cryopreservation of the final product, in-process intermediates, or any hold step.

Stability studies must verify that the storage conditions maintain the quality attributes of the product so that the latter complies with stability specifications. These specifications may differ from release specifications, but they must address product potency. Measuring and calculating the decay of product activity by employing statistical methodologies may require frequent sampling during an extended period and may require analysis of multiple production lots to compensate for the variability of the assays or products.

Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Initial studies are also useful for determining which assays are stability indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of all cell and tissue products, manufacturers should develop assays that address these unique characteristics.

Shipping validations are a special type of stability study with predetermined protocols, testing requirements, and acceptance criteria. Typically, the product is packaged and shipped under normal and extreme conditions, and the material is tested before and after shipping to ensure that it still meets the product release requirements. As described in *Storage and Shipping* (below), special attention must be given to the specific thermal, mechanical, and radiological stresses products will likely encounter.

Stability Challenge Conditions

The stability-indicating profile of a cell or tissue product may vary with time under the influence of a wide variety of environmental conditions, including temperature, mechanical stress, and light. Multifactorial degradation pathways must be considered in the development of a program to investigate the effects of these parameters on product stability. Based on risk assessment, studies should include conditions that are outside of the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation caused by shipping to hot or cold climates, hypobaric conditions in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite.

A short exposure to an environmental condition well outside of an established limit and a long exposure to one just outside of an established limit may be equally detrimental. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should

be investigated if it is scientifically warranted. Special attention should be given to products stored in fluids containing light-sensitive or -reactive components that may give rise to cytotoxic by-products.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to characterize how the product degrades and which assays are stability indicating. For example, if a cell-based product will be held under refrigeration (4°–6°) until use, then studies performed while holding the product for extended times at room temperature (25°) may provide useful information about product integrity as well as the analytical methods used. Such studies should be performed before formal stability studies begin so that the formal studies incorporate the validated stability-indicating assays into the protocol.

STORAGE AND SHIPPING

General Considerations

Storage conditions are chosen to preserve the purity and potency of the product so that the specifications for the product are maintained throughout storage, shipping, and handling at the clinic. Before clinical trial use, initial studies must be conducted to determine acceptable storage, shipping, and handling conditions. The storage conditions and expiration date for the product must be specified. The initial storage and shipping conditions need not be exactly the same as those envisioned for the commercial product, but they should ensure that the product specifications are maintained beyond the initially proposed expiration dating. Once stability-indicating methods are developed and the final container–closure, storage, and shipping conditions are chosen, these conditions must be validated, as discussed under *Stability* (above).

For products with short shelf lives, storage and shipping conditions—even within a single medical center—should be considered together because shipping constitutes the bulk of storage time after manufacturing. The product should be placed in a lightproof, leakproof container with adequate physical support to ensure stability and prevention of leakage during typical conditions of shipment. Special consideration should be given to the ability of gas to permeate the shipping container, especially if the cell-therapy product is stored or shipped on dry ice or liquid nitrogen.

Storage

For each type of cellular therapy product, the manufacturer should establish product storage specifications and acceptable storage conditions, including temperature range or liquid nitrogen level. Storage units require a system that continuously monitors and records temperature and/or liquid nitrogen levels. This includes an alarm system to immediately notify lab personnel of unacceptable storage conditions. The stability of the product during routine storage should be monitored by a stability program (see *Stability*, above).

Cryopreservation is the main mode for the long-term storage of cells. Cellular products are cryopreserved using controlled-rate freezing procedures or equivalent procedures that are known to maintain viability. The temperature of products during freezing and storage should be monitored and documented according to the facility's policies. The stability of the product under the holding conditions at the manufacturing facility and clinical site should also be validated.

The cooling rate for cell solutions during cryopreservation is important because of the mechanical and dehydration injuries resulting from the formation and growth of ice crystals. The ideal temperatures depend on the type of cells and the concentration of the cryopreservative. The optimal cooling rate for most cells is between 1° and 3° per minute. Controlled-rate freezers that can reproducibly duplicate this optimal cooling rate are critical when large numbers of vials or large volumes of cells in bags are being frozen. Once cooled to below freezing, cells should be stored at temperatures below −130°. This can be achieved with electric freezers or with liquid nitrogen.

Storage of cells in the vapor phase of a liquid nitrogen freezer reduces the risk of cross-contamination with other material in the freezer. Freezer equipment should be validated and temperature mapped so that cells are not subjected to temperatures above −130° as the liquid nitrogen evaporates or during freezer opening. Some cells can be stored at −80° if the cells will be used within a few weeks. Cross-contamination can also be prevented by the use of sealed overwraps covering the cryobags.

Many cell-based products cannot be cryopreserved. Because cells continue to metabolize during storage, their expiration period is short—on the order of hours or days. The expiration date can be extended by increasing the volume of storage medium, by adjusting the storage temperature, or by attaching a series of bags or compartments that allow the medium to be exchanged without breaching system sterility.

Storage conditions at the clinical site must also be defined and monitored. Cell-processing centers or clinics involved with bone marrow transplantation generally have liquid nitrogen freezers, but most clinical pharmacies do not. Storage temperatures and characteristics must be defined for each product. The clinical site may need to hold the product in the shipping container until the product can be administered to the patient. If thawing and administering of the cell-based product are performed at the clinic, the laboratory storing or shipping the cells must closely collaborate with the clinic because cells in a concentrated suspension may survive for only a few hours.

Shipping

Shipping containers and shipping procedures need to ensure temperatures are maintained within acceptable ranges for the duration of transportation and under conditions of actual use. These conditions include temperatures within the shipping container, extremes of temperature outside the shipping container (such as those encountered on a hot airport tarmac or in the chilly cargo hold of an airplane), and other shipping challenges (such as x-rays or mechanical vibration). Shipping studies should be conducted during product development in order to identify stresses to which the products may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will protect against extreme temperatures and mechanical stresses.

Most products are shipped by commercial shippers or courier systems. In some cases, critical products are hand-carried onto commercial aircraft. Commercial carriers must obtain special permission in order to bypass scanning by airport x-ray equipment. Special attention should be paid to shipping container labels because both biohazard and patient-specific information may be required in specific areas of the packaging. Shipping validations must be conducted under predefined protocols with predetermined acceptance criteria to ensure that the product meets quality specifications (including potency) once it reaches its final destination.

Cryopreserved cell-based products are typically shipped to medical centers on dry ice or in liquid nitrogen dry shippers. Dry shippers may be preferable because temperature is more readily maintained and monitored. Dry shippers also allow continuous monitoring of the shipper's temperature, which can be collected and logged for up to 14 days. Dry ice and liquid nitrogen are both considered hazardous materials during shipping and must be labeled accordingly.

LABELING

Labeling of cell therapy products is regulated by FDA under 21 CFR 201, 601, 610, and 1271. For biologics, 21 CFR 610 Subpart G outlines the requirements for container and package labeling. When possible, a full label should be affixed to the product container. This includes the proper name of the product obtained from the US Adopted Names (USAN) Council; the name, address, and license number of the manufacturer; the lot number; the expiration date; for multiple-dose containers, the recommended individual dose; the statement "Rx Only"; instructions to the dispenser to provide a Medication Guide, if one is required, to each patient; and if the container is not enclosed in a package, all items required for a package label. If the label is too small to accommodate all this information, a partial label can be used, but the following information must appear on the partial label: the name expressed either as the proper or USAN name; the lot number and the name of the manufacturer; and for multiple-dose containers, the recommended individual dose. When partial labels are used, the container must be placed in a package that contains a label bearing all the items required for the package label. For containers that cannot accommodate any label, the container must be placed in a package that bears all the information required for a package label. In addition, when affixed to the container the label should not impede inspection of the contents. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life.

The package label must contain the proper or USAN name of the product, the name, address, and license number of manufacturer, the lot number, the expiration date, the preservative used and its concentration, or the words "No Preservative" if appropriate, the number of containers if more than one, the amount of product in the container, the recommended storage temperature, the words "Shake Well," "Do Not Freeze," or other instructions as indicated, the recommended individual dose for multi-dose containers, the route of administration, known sensitizing substances, the type and amount of antibiotics added during manufacture, inactive ingredients if they are a safety factor, the adjuvant, the source of the product when this is a factor for safe administration, minimum potency expressed in terms of official standard for potency or the statement "No U.S. Standard of Potency," and finally the statement "Rx Only." Regulations in 21 CFR 610.62 regard the position and prominence of the proper or USAN name in relation to a trade name.

Additional labeling requirements apply because cellular therapy products are also considered HCT/Ps in 21 CFR 1271.90. For autologous cell therapies, the manufacturer is exempt from the requirements of determining donor eligibility. However, if the recommended testing for pathogenic or microbial contaminants is not performed before release, the label must contain the statement "FOR AUTOLOGOUS USE ONLY" or "NOT EVALUATED FOR INFECTIOUS SUBSTANCES." The label must also contain the Biohazard legend shown in 21 CFR 1271.3(h) with the statement "WARNING: Advise patient of communicable disease risks." For patient-specific products, the patient's full name, initials, or a combination of these must appear on the labeling to ensure that the product will be administered to the appropriate patient.

In addition, regulations govern the content and format of labeling for human prescription drug products (including biological products), otherwise known as the package insert. These regulations, which apply to approved therapeutics, went into effect 30 June 2006. Details about this content and format can be found in 21 CFR 201.56 and 201.57. These changes were designed to enhance the ability of the health care practitioners to access, read, and use prescription drug labeling. The main change is the addition of a half-page highlights section. Otherwise most of the changes involve rearrangement of sections to move to the front the most critical information for prescribing.

In addition to the specific FDA regulatory requirements, several groups have designed ISBT 128, a standard for uniform labeling of cellular therapy products, where ISBT stands for International Society of Blood Transfusion and the number 128 reflects the choice of barcode symbology known as Code 128. ISBT 128 defines the data structures and the placement of bar

codes and their corresponding eye-readable text that appears beneath the bar code. In addition, this standard provides class names for different types of cellular products, modifier text for cell processing, manipulation text for how the cells are manufactured, cryoprotectant text for frozen cell products, and various other texts that must go on the labels. Although this voluntary standard meets different organizations' requirements for labeling cellular products, it does not currently meet FDA regulatory requirements. Consequently, labels that comply with ISBT 128 must be supplemented with additional information required by FDA.

CONSIDERATIONS FOR TECHNOLOGY TRANSFER

Transfer of the skills, knowledge, technologies, and methods of manufacturing necessary to create a cell or tissue-based product is essential to ensure that scientific and technological developments are accessible to users who can then further develop and advance the technology into new products, processes, applications, materials, or services. Some general considerations for technology transfer activities are summarized below.

The process of developing a cellular or tissue-based therapy is complex and often involves several rounds of technology transfer throughout the product's life cycle. Some examples of technology transfer activities include: from bench research to translational research; transfer from research and development to GMP-compliant manufacturing; and change in manufacturing facility (for example, from in-house manufacturing to a contract manufacturer).

Manufacturers should anticipate the need for technology transfer during the research and development stage of a cell or tissue-therapy process. This should result in good documentation practices for product research and development, including testing procedures. Data and results should be retained in the format of development reports or technical reports to provide historical information that can be referenced and used in regulatory filings. Critical raw materials, procedures, and equipment should also be identified during technology transfer. Product and process development progress should be monitored against milestones established as part of risk assessment and gap identification in the technology transfer plan. *Table 3* provides an overview of the steps involved in technology transfer.

Table 3. Technology Transfer—Fundamental Steps

Preparation	• Define the scope, strategy, and risks associated with the project that will be transferred • Identify overall gaps and process transferability • Assess availability of documentation such as manufacturing and testing procedures, sampling plans, in-process and final product data and specifications, material specifications (including source, testing requirements, and quantities required for a manufacturing procedure or test procedure), equipment specifications, specialized training requirements, facility requirements, and infrastructure requirements • Establish a governance body consisting of leads, experts, and mentors from both the sending and receiving sides; determine responsibilities for each group and individual • Define communication and reporting channels • Identify performance measurements, milestones, and timelines
Development and Implementation	• Establish a risk management plan • Establish a technology transfer master plan • Develop a training plan • Establish documents at the receiving site (specifications, SOPs, batch records, and standard test methods) • Train operations, quality, and support personnel for sustainability • Qualify materials and vendors • Establish and execute equipment comparability/suitability protocols • Calibrate equipment at receiving site • Qualify personnel, equipment, and facility at receiving site (includes execution of aseptic process validations, sterile media fills, and cleaning validations) • Establish and execute methods/assay qualifications • Establish a product stability program • Perform engineering and consistency/qualification runs • Assess need to establish comparability and prepare regulatory filings • Establish and execute shipping qualifications
Maintenance	• Collect and trend process/product data • Monitor product stability • Manage change control • Train and requalify personnel • Recalibrate and requalify equipment • Update regulatory filings

The ultimate goal of technology transfer is for the recipient to consistently reproduce a process to make comparable product in compliance with regulations. It is not atypical for manufacturers to develop and implement process improvements during early stages of technology transfer to support scale-up and manufacturing for Phase I/II clinical trials. However, during technology transfer for Phase III studies, pivotal trials, or commercial manufacturing, changes to the process or product should be avoided because they could require additional clinical studies and adversely affect time to market.

General Chapters

REGULATIONS AND STANDARDS

The Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Public Health Service Act (PHS Act) provide the legal framework for FDA regulation of biological products, including cell-based therapy products. A list of frequently used terms in regulation of cellular-therapy products is presented in *Table 4*. In 1993 FDA provided notice that it intended to regulate cellular and gene-therapy products as biological products (Federal Register 1993;58:53248–53251). FDA defined somatic cell therapy products as autologous (i.e., self), allogeneic (i.e., intraspecies), or xenogeneic (i.e., interspecies) cells that have been propagated, expanded, selected, pharmacologically treated, or otherwise altered ex vivo for administration to humans for the prevention, treatment, cure, diagnosis, or mitigation of disease or injuries. For other biological products and drugs, clinical trials involving somatic cellular therapy products must be initiated under an investigational new drug (IND) application. After a sponsor submits sufficient evidence of product safety and clinical effectiveness, FDA approval can be obtained for marketing in the form of a biologics license application (BLA) or PMA.

As defined by FDA, cellular therapy products are considered to be drugs, biological products but also HCT/Ps that are regulated under Section 351 and/or Section 361 of the PHS Act. This means that cell-based therapies are subject to cGMP (21 CFR 210 and 211), Biologics Product regulations (21 CFR 610), and HCT/P regulations (21 CFR 1271) including cGTP.

In recent years FDA has issued a number of regulations and guidance documents for human cell and tissue products (see *Appendix* and www.fda.gov/cber/). Of particular importance are the regulations at 21 CFR 1271 that establish a tiered, risk-based approach for HCT/Ps. In this regulatory framework, many conventional human cells or tissues are not subject to premarket approval and have only to comply with GTPs, including donor eligibility. This lower tier of regulatory oversight is intended to prevent to the introduction, transmission, or spread of communicable disease. When human cells or tissue are the starting material for the creation of a novel cell-based product, additional regulatory requirements are applicable. This higher tier of regulatory oversight includes compliance with GMPs, biological product standards, and premarket approval (see 21 CFR 1271.10). In almost all cases, the cell-based products described in this general chapter should comply with the higher tier of regulatory oversight.

In addition to cellular therapy-specific regulations and guidance, many general guidelines such as those related to aseptic processing, GMP expectations during development, process validation, and others are relevant and applicable (see www.fda.gov). Additionally, ICH has issued guidance documents for qualifying cell and tissue-based products (see *Appendix* and www.ich.org). Some of the guidelines and concepts in these documents are reproduced in *USP–NF*.

The regulatory pathway for cellular-therapy products parallels that of pharmaceuticals, and as the product moves from early research through pivotal trials and finally marketing approval, the degree of manufacturing control becomes increasingly stringent. This has implications for the manufacturing unit and may dictate that the site be moved. Standards-setting organizations encourage the use of a fully functional quality unit to oversee manufacturing progress. Information is available on the FDA Web site, along with references to groups charged with guiding the medical community and the manufacturing unit during development.

In addition to USP general chapters and monographs for cell and tissue-based therapies, a number of professional standards-setting organizations (see *Table 5* and *Appendix*) have worked closely with regulatory authorities to develop standards and practices. These organizations ensure that standards are current and comply with governmental regulations. Such standards are a supplemental source of knowledge in identification of donors, donor screening and testing, product collections, processing of cellular products, administration, adverse event reporting, and follow-up after treatment. AATB has developed guidelines for sourcing allogeneic tissue. Over the years various organizations have tried to harmonize standards, including the development of common information circulars that can be compared with package inserts. At present, however, compliance with one organization's standards does not ensure compliance with those of any other organization.

Many benefits accrue to manufacturing facilities that participate in voluntary standards programs. Professional standards-setting organizations participate in educational workshops and disseminate information about operational issues. They also maintain close surveillance of FDA activity and training of inspectors. Further, FDA relies on accreditation by voluntary standards program, and FDA's unannounced inspections have led to an increasingly high level of compliance in laboratory and clinical settings and has also undoubtedly increased patient safety. Third-party payors and hospital-ranking services have begun to use accreditation reports in their evaluation of quality programs.

Table 4. Frequently Used Terms in Regulation of Cellular-Therapy Products

TERM	DEFINITION
351 products	Regulated under Section 351 of the PHS Act
361 products	Regulated under 21 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products
BLA	Biologics Licensure Application
CBER	Center for Biologics Evaluation and Research
CDRH	Center for Devices and Radiologic Health
GMPs	Good Manufacturing Practices
GTP	Good Tissue Practices, 221 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products

General Chapters

Table 4. Frequently Used Terms in Regulation of Cellular-Therapy Products *(Continued)*

TERM	DEFINITION
IDE	Investigational Device Exemption. An investigational device exemption (IDE) allows the investigational device to be used in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PA) application or a Premarket Notification [510(k)] submission to FDA.
IND	Investigational New Drug. An IND is a request for FDA authorization to administer an investigational drug to humans. IND regulations are contained in 21 CFR 312.
PMA	Premarket approval

Table 5. Cellular Therapy Product Standards-Setting Organizations

AABB	AABB, formerly known as the American Association of Blood Banks, is an international association representing individuals and institutions involved in activities related to transfusion and cellular therapies, including transplantation medicine.	http://www.aabb.org/
AATB	The American Association of Tissue Banks is an educational and scientific, tax-exempt organization that facilitates the provision of transplantable tissues of uniformly high quality to meet national needs. AATB publishes standards to ensure that the conduct of tissue banking meets acceptable norms of technical and ethical performance. AATB conducts an accreditation program for establishments that retrieve, process, store, or distribute human tissue for transplant. A certification program is administered for tissue-bank personnel toensure that tissue-banking activities are performed in a professional manner consistent with the standards of the association.	http://www.aatb.org/
ASTM	ASTM International (ASTM), originally known as the American Society for Testing and Materials, is one of the largest voluntary standards-development organizations in the world and provides technical standards for materials, products, systems, and services. ASTM International standards are used in the information infrastructure that guides design, manufacturing, and trade in the global economy.	http://www.astm.org/
FACT	The Foundation for the Accreditation of Cellular Therapy is a nonprofit corporation co-founded by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT) for voluntary inspection and accreditation in the field of cellular therapy.	http://www.factwebsite.org/
NMDP	The National Marrow Donor Program is a nonprofit organization that operates the federally funded registry of volunteer hematopoietic cell donors and umbilical cord blood units in the United States.	http://www.nmdp.org/
ICCBBA	The International Council for Commonality in Blood Banking Automation was established and given the responsibility for implementation and management of the ISBT 128 standard, a system for identification, labeling, and processing of human blood, tissue, and cellular-therapy products using an internationally standardized system.	http://www.iccbba.org/

GLOSSARY

Adventitious Agent: A foreign material that is introduced inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

Allogeneic: From an unrelated member of the same species but with a different genotype.

Ancillary Materials: Components used during manufacturing that should not be present in the final product. Examples: growth factors, cytokines, monoclonal antibodies, cell-separation devices, and media components.

Apheresis: Procedure of withdrawing blood from a donor, removing select components (e.g., platelets or leukocytes), and transfusing the remainder into the donor.

Autologous: From one's own body.

Bioassay: Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency.*)

Biological Product: Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries in humans. (The term analogous product has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

Biotechnology: Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

B Lymphocytes (B Cells): A class of lymphocytes that produce antibodies and are derived from bone marrow.

Bone Marrow Cells: A variety of undifferentiated cells (stem cells) and differentiated cells (lymphocytes, granulocytes, erythrocytes, and platelets) found in the internal cavities of bones or bone marrow.

Bone Marrow Transplantation: Transplantation of bone marrow cells that are capable of maintaining the hematological functions indefinitely. Technique used in the treatment of immunological disorders (severe combined immune deficiencies such as ADA deficiency), hematological disorders (anemia), metabolic disorders (Gaucher disease), and malignant diseases (leukemia, lymphoma, or solid tumor).
CD34: Cluster of differentiation cell-surface marker 34. CD34 is a protein that distinguishes stem and progenitor cells from more mature blood cells.
Cell Lines: Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or be immortalized (made to replicate indefinitely).
Cellular Therapy: Therapy that uses whole cells to treat a disease, condition, or injury.
cGMP: Current good manufacturing practice.
Chondrocytes: Cells that produce the components of cartilage.
Clonal: Genes, cells, or entire organisms derived from and genetically identical to a single common ancestor gene, cell, or organism.
Clonogenic Assay: Procedure based on the ability to give rise to a clone of cells.
Combination Products: Therapeutic products that combine drugs, devices, and/or biological products.
Cytokine: Any factor that acts on cells; usually a protein that promotes growth.
Cytoplasm: Cellular material that is within the cell membrane and surrounds the nucleus.
Cytotoxic: Able to cause cell death.
Culture Medium: The liquid that covers cells in a culture vessel and contains ingredients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.
Dendritic Cells: Cells that sensitize T cells to antigens.
Differentiation: A process of biochemical and structural changes by which cells become specialized in form and function.
ELISA: Enzyme-linked immunosorbent assay. An immunoassay that uses an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.
Embryonic Stem Cell, Human (hESC): Stem cell derived from the inner cell mass of the blastocyst.
Endothelial Cells: Epithelial cells of mesodermal origin that line the internal cavities of the body, such as heart and blood and lymph vessels.
Engraftment: Process whereby cells, tissues, or organs are implanted or transplanted into another organism. Refers both to the mechanical and the biological processes necessary to have a fully functional graft.
Epidermal: Pertaining to the outermost and nonvascular layer of the skin derived from embryonic ectoderm.
Epithelial Cells: Cells from the linings of various organs, e.g., respiratory, intestinal, or vascular epithelial cells.
Ex Vivo: Outside of the living body. Refers to a medical procedure in which an organ, cells, or tissue are taken from a living body for a treatment or procedure, and then returned to the living body.
Feeder Cells: Cells used in co-culture to maintain pluripotent stem cells. For hESC, typical feeder layers include mouse embryonic fibroblasts or human embryonic fibroblasts that have been treated to prevent them from dividing.
Fibroblasts: Connective tissue cells that have the capacity to produce collagen.
Fluorescence-Activated Cell Sorter (FACS): A machine that sorts cells based on fluorescent markers attached to them.
Formulated: Prepared in accordance with a prescribed method or conditions.
Graft-versus-Host Disease: Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.
Granulocyte: One of three types of white blood cells. These cells digest bacteria and parasites.
Granulocyte–Macrophage Colony-Stimulating Factor (GM CSF): A natural hormone that stimulates white blood cell production, particularly that of granulocytes and monocytes.
Growth Factors: Factors responsible for regulatory cell proliferation, function, and differentiation.
Hemacytometer: A device used to manually count cells.
Hematopoietic: Pertaining to or affecting the formation of blood cells.
Hematopoietic Stem Cells: Stem cells that give rise to all red and white blood cells and platelets.
Hepatocytes: The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells are generally not dividing, but when injured they can divide and regenerate until the injured cells are replaced.
Human Leukocyte Antigen (HLA): Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.
Immunoassay: Technique for identifying substances based on the use of antibodies.
Immunofluorescence: Technique that combines an antibody detection strategy with a fluorescent label for visualization often used in combination with microscopy or fluorescence activated cell sorting.
Immunogenic: Substance capable of inducing an immune response; a form of antigen that induces an immune response, as opposed to a tolerogen that induces tolerance.
In Vivo: Procedure performed in the living organism.
In Vitro: In the laboratory (outside the body). The opposite of in vivo (in the body).
Islet Cells: β-islet cells of the pancreas that secrete insulin.
Keratinocytes: Differentiated epidermal cells that constitute the top layer of cells in the skin.

Lineage (Committed Progenitor Cells, Differentiated Cells): Specific path of cell differentiation that can be traced to a single cell of origin.
Macrophage: Any of many forms of mononuclear phagocytes that are found in tissues and arise from hematopoietic stem cells in the bone marrow.
Mesenchymal Stem Cells: Multipotent stem cells that can differentiate into a variety of cell types.
Monoclonal Antibodies: Antibodies that are derived from a single cell clone.
Myocytes: Fundamental cell units in the muscle. Target cells for insertion of genes that encode secretory proteins.
Natural Killer Cells (or NK Cells): Cytotoxic lymphocytes that constitute a major component of the innate immune system.
Neuronal Stem Cells: Stem cells found in neural tissue that can give rise to neurons and glial cells.
Osteogenic Cells: Derived from or involved in the growth or repair of bone.
Passage: The process in which cells are disassociated, washed, and seeded into new cultures after a round of cell growth and proliferation. The number of passages is a good indication of the age of the cultures and expected stability.
Process Validation: Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.
Polymerase Chain Reaction (PCR): Technique to amplify a target DNA or RNA sequence of nucleotides by cycles of polymerase-based copying, resulting in geometric increases in copy number.
Potency: A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.
Progenitor Cell: Parent or ancestral cell, usually one that is already committed to differentiate into a specific type or lineage of cells.
Regenerative Medicine: An emerging interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function using a combination of approaches including, but not limited to, the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering, and the reprogramming of cell and tissue types.
Serum-Free: Refers to cell growth medium that lacks a serum component.
Somatic Cells: Cells other than the germ cells.
Stem Cell: Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.
Supravital Dye: A dye that stains only living cells.
Suspension Culture: Growth, in suspension, of cells not requiring attachment to substrate in order to undergo cell division.
T Cells: Lymphocytes that acquire functional repertoires and the concept of self in the thymus and are responsible for cell-mediated immunity. There are several subsets of T cells (helper T cells, suppressor T cells, and cytotoxic T cells).
Umbilical Cord Blood Stem Cells: Stem cells derived from the blood that remains in the placenta and in the attached umbilical cord after childbirth.
Undifferentiated Cells: Cells that have not yet developed into a specialized cell type or tissue.
Xenogeneic: From a different species.
Xenotransplantation: Transplantation of organs from one species to another (e.g., from pigs to humans).

APPENDIX

List of Relevant Regulatory References

Cellular therapies and cell-therapy components are regulated by FDA as biological products. The general requirements are listed in national laws and international guidance. In the United States, national requirements are codified in different sections of 21 CFR, and additional recommendations are available in FDA guidance documents. International guidance documents are available from ICH, the European Agency of Medicines (EMA), and the World Health Organization (WHO). Although guidance documents from ICH are well referenced in this general chapter, those from WHO and EMEA are not, and manufacturers of cellular or tissue-based products intended for markets outside the United States are advised to refer to relevant guidances from relevant nations. Beyond USP chapters referenced in this chapter, the following list includes regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance:

Code of Federal Regulations (CFR)

- 21 CFR 201.56–57
- 21 CFR 210
- 21 CFR 211
- 21 CFR 600.3
- 21 CFR 601
- 21 CFR 610
- 21 CFR 820
- 21 CFR 1271
- 21 CFR 46

FDA Guidance Documents

- *Draft Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*
- *Draft Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*
- *Draft Guidance for Industry: Current Good Tissue Practice (cGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*
- *Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, February 2007. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm073964.htm
- *Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans*, April 2003. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm074354.htm
- *PHS Guideline on Infectious Disease Issues in Xenotransplantation*, January 2001. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm074727.htm
- *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production*, October 2006. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070287.pdf
- *Guidance for Industry: Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene-Therapy Products*, CBER, 2008. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072612.htm
- FDA Blue Book G95-1, http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080735.htm

National and International Regulatory Documents

- The United States Consensus Standard for the Uniform Labeling of Cellular Therapy Products using ISBT 128, available at: http://www.iccbba.org/usconsensusstandard_cellulartherapy.pdf
- ISO 10993-1:2003, Biological evaluation of medical devices—Part 1: Evaluation and testing, available at: http://www.iso.org
- ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology, available at: http://www.ich.org
- ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products, available at: http://www.ich.org
- ICH Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, available at: http://www.ich.org
- ICH Q9: Quality Risk Management, available at: http://www.ich.org
- Naming Scheme for Cell Therapies by the United States Adopted Names (USAN) Council, available at: http://www.ama-assn.org/
- Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), available at: http://www.nap.edu/

⟨1047⟩ GENE THERAPY PRODUCTS

INTRODUCTION

Gene therapy products allow administration of nucleic acids to modify the genetic material of cells. Gene therapy products can be broadly classified based on the approach to delivery and include the following: (1) viral vectors [viruses that harbor the gene(s) of interest but usually without the mechanism to self-replicate in vivo]; (2) nucleic acids in a simple formulation (naked DNA); and (3) nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Where introduction of nucleic acid to cells takes place ex vivo, the cell population that is administered becomes the gene therapy product. Guidance specific to the manufacturing, processing, characterization, and administration of cell-based products is provided in *Cellular and Tissue-Based Products* ⟨1046⟩.

Decisions regarding the choice of a gene vector can be complex (see *Design Considerations for Gene Vectors* under *Manufacturing of Gene Therapy Products*). The viruses most commonly used include murine retroviruses, human adenoviruses, and human adeno-associated viruses (AAVs). It is inherent in the definition of gene therapy in this chapter that the administration of nucleic acid through transduction is expressed as RNA and then as protein. Examples of gene therapy products are shown in *Table 1*.

General Chapters

Table 1. Examples of Gene Therapy Products

Categories or Strategies	Indication: Administered Product
Gene replacement Short-term Long-term	Cardiovascular disease: growth factor vector on a biocompatible scaffold[a] Cystic fibrosis: transmembrane conductance regulatory vector Hemophilia: factor VIII or IX vector
Direct cell killing	Cancer: recombinant oncolytic viruses
Immunotherapy	Cancer: autologous tumor cells transduced with cytokine or other immunomodulatory genes; lymphocytes transduced with receptors for tumor antigens Arthritis: gene-modified autologous lymphocytes
Conditionally lethal genes[b]	Cancer (solid tumor): thymidine kinase (TK) or cytosine deaminase (CD) vector into tumor cells Graft-versus-host disease (GVHD): TK or CD vector transduced into donor T cells
Gene disruption via antisense RNAs, ribozymes, and inhibitory RNAs expressed via a vector	Cancer: anti-oncogene vector Cytomegalovirus retinitis: anti-viral vector Human immunodeficiency virus (HIV): autologous lymphocytes transduced with antiviral ribozyme vector
Intrabodies	Cancer or HIV: vector encoding single-chain antibody to a tumor protein or a viral protein, respectively

[a] This product promotes formation of new blood vessels.
[b] Cells with conditionally lethal genes as well as their neighboring cells are killed after the administration of a second drug in vivo. For TK, the drug is gancyclovir. For CD, the drug is 5-fluorocytosine.

CHAPTER PURPOSE AND ORGANIZATION

Clinical uses for gene therapy products, their manufacturing processes, and analytical schemes for determining identity, dose, potency, purity, and safety are rapidly evolving, and are as diverse as the products themselves. This chapter summarizes the issues and best current practices in the manufacturing, testing, and administration of gene therapy products. Usually *USP* chapters focus on materials that are commercially available. This chapter, however, not only discusses products for commercial applications, but it also addresses the production of clinical trial materials. When different approaches are options for clinical trial material compared to those used for commercial product, this is discussed.

Where appropriate, reference is made to applicable guidance including International Conference on Harmonization (ICH) quality guidelines because the principles apply even though gene therapy products may be outside the official scope. A list of regulatory and guidance documents applicable to gene therapy is presented in the *Appendix*, together with a list of terms commonly used in the gene therapy field. The traditional compendial perspective is to develop public standards that can be applied to a particular final product without providing production details. This chapter attempts to specify when traditional methodologies or standards can be adapted.

This chapter is extensive because of the diverse nature of the products and the special considerations that they require. Manufacturing has been divided into two sections: the first discusses general aspects of manufacturing and process development, and the second discusses vector design and class-specific topics. *On-Site Preparation and Administration* follows the manufacturing sections because the handling of these products at the clinic often requires facilities and expertise not found in a typical hospital. Other manufacturing-related sections include: *Analytical Methods*; *Stability*; *Storage and Shipping*; and *Labeling*. The section *Regulations and Standards* summarizes existing guidelines and highlights the need for the development and validation of new methodologies to assess product quality. The *Glossary of Terms* lists and defines the terms and abbreviations used in this chapter and those commonly employed in this field.

MANUFACTURING OVERVIEW

Introduction

The manufacturing of gene therapy products has been divided into two sections. This section, *Manufacturing Overview*, discusses five topics that apply to manufacturing of all gene therapy products: (1) raw materials, (2) characterization of banked materials, (3) in-process controls, (4) specifications, and (5) validation considerations. The second section, *Manufacturing of Gene Therapy Products*, addresses manufacturing of gene therapy vectors, both viral and nonviral, and discusses the design of gene vectors in detail.

All the general principles of current good manufacturing practice (cGMP) outlined by FDA in 21 CFR 210, 211, 600s (especially 21 CFR 610), and 820, as well as other *USP* chapters apply to the manufacturing of gene therapy products. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of cGMP. cGMPs are applied throughout clinical development. Typically, the extent of control increases as clinical development progresses, and full cGMP compliance is expected by initiation of manufacturing in support of Phase III clinical trial(s). The facility and equipment should be carefully designed, built, and validated to support the manufacturing process and to maintain the required product/facility segregation. Preventive maintenance and calibration should be performed routinely on

critical equipment. Incubators, bioreactors, and freezers should be fitted with alarm systems that can remotely signal failure. Quality systems should be established to ensure manufacturing is consistent and in control. Systems include but are not limited to the following: change control, document control, environmental monitoring, training, validation master plans, raw material testing and release, vendor approval, product testing and release, stability testing, and corrective/preventive action (CAPA).

Ancillary Materials

A wide variety of raw materials, including ancillary materials, may be used in manufacturing. Raw materials may include complex substances such as cells, tissues, biological fluids, growth factors, and monoclonal antibodies. Some of these materials may remain in the final therapeutic product as active substances, cryoprotectants, or excipients. An ancillary material exerts an effect on a therapeutic material (for example, a cytokine may activate a population of cells) but is not intended to be present in the final therapeutic product. The quality of raw materials used in the production of a gene therapy product can affect the safety, potency, and purity of the product. Therefore, qualification of this type of materials is necessary to ensure the consistency and quality of all gene therapy products. Activities involved with raw material qualification will change as products move through various stages of clinical development and on to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. A qualification program for raw materials used in the manufacturing of gene therapy products should address each of the following areas: (1) identification and selection, (2) suitability for use, (3) characterization, (4) animal-derived components, and (5) quality assurance. For all raw materials, it must be considered when and where each is used in the manufacturing process because this can help define selection criteria. *USP* chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩ should be consulted for specific information about implementing an appropriate qualification program for these materials. Other *USP* chapters provide information about the qualification and standards of specific ancillary materials (e.g., *Bovine Serum* ⟨1024⟩, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* ⟨90⟩, and *Growth Factors and Cytokines Used in Cell Therapy Manufacturing* ⟨92⟩).

Characterization of Cell and Virus Banks

CELL BANKS

A cell bank is a collection of vials containing cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single cell clone. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although more tiers are possible. The MCB is manufactured in accordance with cGMP and preferably is obtained from a qualified repository source (source free from adventitious agents) with known and documented history. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to consistency of production of clinical or licensed product batches because the starting cell material is always the same. Cell banks used for the preparation of virus banks or clinical product should be suitably characterized before use. Aspects of cell banking and validation are addressed in *Cellular and Tissue-Based Products* ⟨1046⟩, *Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of rDNA-Derived Protein Products* ⟨1048⟩, and *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩.

VIRUS BANKS

The master virus bank (MVB) is similar in concept to the MCB because it is derived from a single production run and is uniform in composition. The working virus bank (WVB) is derived directly from the MVB. As with the cell banks, the purpose of a virus bank is to have a consistent source of virus that is shown to be free of adventitious agents for use in production of clinical or product batches. In keeping with cGMP regulations, testing of the cell bank that will be used for production of the virus banks, including quality assurance testing, should be completed before the use of this cell bank for production of virus banks.

QUALIFICATION

Cell and viral bank characterization is an important step toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. Testing to qualify the MCB or MVB is performed once, and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB or MVB should be established. It is important to document the MCB and MVB history, the methods and reagents used to produce the bank, and the storage conditions. All the raw materials required for production of the banks—media, sera, trypsin, and similar substances—must also be tested for adventitious agents.

QUALIFYING THE MASTER CELL BANK

The FDA *Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy* (March 1998) provides specific recommendations for qualifying MCBs. Additional guidance is provided in ICH Q5D. A description and history of the cell line is required, along with a description of the freezing process, storage conditions, and the number of vials prepared. The identity of the cells should be analyzed by genotypic and/or phenotypic markers. For MCB containing vector sequences, the presence and integrity of the vector should be demonstrated using molecular assays (restriction endonuclease mapping and/or nucleic acid sequencing) and/or measurement of vector gene expression. Purity must be analyzed to exclude bacterial, mycoplasma, fungal, and viral contamination (other than vector sequences). Freedom from adventitious viruses should be demonstrated using both in vitro and in vivo virus tests and appropriate species-specific tests such as the mouse antibody production (MAP) test. Special attention should be given to the detection of replication-competent virus (RCV) arising from recombination of the vector and viral sequences. The MCB is further qualified by tests conducted on cells (from the MCB or WCB) expanded to the limit of in vitro cell age for production.

QUALIFYING THE MASTER VIRUS BANK

Testing of the MVB is similar to that of the MCB and should include testing for freedom from adventitious agents in general (such as bacteria, fungi, mycoplasma, or viruses) and for organisms specific to the production cell line, including RCV. Identity testing of the MVB should establish the properties of the virus and the stability of these properties during manufacture.

QUALIFYING THE WORKING CELL OR VIRUS BANK

Characterization of the WCB or WVB is generally less extensive and requires the following: (1) testing for freedom from adventitious agents that may have been introduced during generation of the WCB, (2) testing for RCV, if relevant, (3) routine identity tests to check for cell line cross-contamination, and (4) demonstration that aliquots can consistently be used for final product production. This assumes that the WCB and WVB were prepared in a controlled environment using media and equipment that were screened appropriately for adventitious agents. If not, additional release testing is required.

In-Process Controls

Manufacturing processes should have well-defined go–no go decision criteria that are applied to key in-process intermediates and are used to pool material that has been processed through a step in several sublots. Quality must be built into the product as well as tested during batch release. In-process controls are the assays or tests that are performed to ensure that the in-process intermediate is of sufficient quality and quantity to ensure manufacture of a quality final product. Examples of in-process controls are listed in *Table 2*. The main reason for performing the in-process control is to ensure that the correct product with anticipated quality and yield is obtained. Intermediate in-process material that fails to satisfy the in-process control criteria should not be used for further manufacturing. This material may be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications before it can undergo further manufacturing. If several sublots will be pooled for further processing, sublots that fail to satisfy the criteria should not be included in the pool, even if the pool containing these failed sublots would pass the in-process assay criteria. During clinical development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. The information from these runs is also used to set the criteria for the selected assays. In-process controls are performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended, and actions should be taken to correct problems as they arise.

Table 2. Examples of In-Process Control Applications

Type of Product	Attribute to Control
Viral gene therapy	Quantity of virus after virus culture Specific activity of virus in fractions after column chromatography Quantity of host-cell DNA in fractions after column chromatography
Nonviral gene therapy	Optical density or change in oxygen consumption during culture Amount and form of plasmid before culture harvesting Amount and form of plasmid after extraction steps Amount of pyrogen or endotoxin after extraction steps in plasmid pool

Specifications

The specification for a gene therapy product should be chosen to ensure the safety and efficacy of the product before use. Selected tests should be product specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifi-

cations for cell and gene products should follow the principles outlined in ICH Q6B and should reflect the FDA *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs).*

Establishing specifications for a drug substance and drug product is part of an overall manufacturing control strategy that includes control of raw materials, excipients, and cell and virus banks; in-process testing; process evaluation and validation; stability testing; and testing for consistency of lots. When combined, these elements provide assurance that the process is in control and that the key quality attributes of the product are maintained. Appropriate specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Specifications for each product and its ingredients should be developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to safety and efficacy assessments. Specifications should accommodate the inherent variabilities exhibited by the production process and by the assay. Some lot-release specifications typically applied to biologics may require re-examination for these product types.

The procedures in a specification for the product are anchored by appropriate reference standards. The reference standard for the product ensures that the product, as measured by the release assays, does not change significantly over time. The reference standard is manufactured using the same process as used for clinical production and is subject to all in-process and final release testing. In addition, the reference standard may be subjected to additional characterization not typically performed as part of lot release. The reference standard need not be stored at the same dose, formulation, or temperature as the product, but the stability of this reference standard should be determined. The reference standard verifies that a test produces acceptable results (passes its system suitability tests). A specific assay standard (working standard) can be used in the test, but it should be calibrated against and behave like the reference standard. Changing to a new reference standard (lot) should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted.

Additional specifications may be needed to produce a safe and effective gene therapy product. These might relate to some of the controls and action limits used to maintain standards and consistency for raw materials, excipients, and the manufacturing process (see *Ancillary Materials* and *In-Process Controls*). Specifications should be established to allow acceptance of raw materials and excipients used in the final formulation of the product. In addition, tests should be performed at critical decision steps during manufacture or at points where data serve to confirm consistency of the process. In-process release specifications should be established for each control step. Heterogeneity can result from the manufacturing process or storage of the product. Therefore, the manufacturer should define the pattern of heterogeneity within the product and should establish limits that will maintain the therapeutic efficacy and safety of the product.

In some cases, specifications may be established for lot release as well as for shelf life. As discussed in ICH Q5C and *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* 〈1049〉, the use of different specifications should be supported by sufficient data to demonstrate that the clinical performance is not affected. Acceptance criteria should be established and justified on the basis of data obtained from lots used in preclinical and clinical studies and lots used for demonstration of manufacturing consistency and on the basis of relevant development data such as those arising from validated analytical procedures and stability studies. Acceptance criteria should also be correlated with safety and efficacy assessments.

Once specifications have been established, test results should be trended. Results that are out of specification (OOS)—or even those that are out of trend—should be investigated before the material is considered for further processing. The purpose of an investigation is to determine the cause of the discordant result. FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that the product is not within the specification, the lot should be rejected. In unique situations, a product that does not meet all specifications may have to be administered to a patient. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results.

Considerations for Validation

The potential for wide biological variation in gene therapy products, particularly for patient-specific treatments, affects the validation effort. Nevertheless, the basic principles of process validation for any biological product, including recommendations by ICH, FDA guidance documents, *Validation of Compendial Procedures* 〈1225〉, and *Validation of Microbial Recovery from Pharmacopeial Articles* 〈1227〉, apply to the validation of most gene therapy products. Guidelines for validating viral vaccines can be relevant to gene therapy processes that produce viral vectors. The hold steps in a manufacturing process should be validated to ensure that in-process intermediates are within specification and that the quality attributes of the final product are maintained. Product-release assays should be validated before production of the materials for Phase III pivotal clinical trials.

Process validation demonstrates that the unit operations of the manufacturing process perform consistently and can generate a quality product that meets specifications. Because biological processes are prone to variability, the consistency and ro-

bustness of the manufacturing process should be determined by validating the process on at least three lots. Process validation issues pertinent to cell-based products are addressed in ⟨1046⟩.

If possible, the process should be validated for virus clearance according to principles discussed in ICH Q5A. If this is difficult because of the nature of the gene therapy vector (e.g., enveloped virus), additional characterization of cells and animal-derived components used in the production process should be considered. If the gene therapy product is manufactured in a multiproduct facility, validate cleaning of multiproduct equipment and rooms to demonstrate the effectiveness of cleaning agents to inactivate or remove virus.

MANUFACTURING OF GENE THERAPY PRODUCTS

Introduction

Principles for the production of pharmaceutical or biological products are also relevant to the production of gene therapy vectors for use in humans. The same cGMP requirements are applied to ensure that a high-quality product is delivered to the patient. Because of the nature of gene therapy manufacturing systems, most manufacturers face development issues such as scalability, yield, cost efficiency, and product stability.

Most gene therapy vectors have been produced only in relatively small batches necessary to meet the needs of early clinical trials conducted in small numbers of patients. However, the promise of gene therapy in larger patient populations has led to progress in large-scale production and purification technology. This section focuses on designing vectors for gene therapy and choosing a suitable production technology.

Design Considerations for Gene Vectors

TYPES OF VECTORS

A typical gene therapy vector is composed of the following: (1) the vector backbone; (2) a promoter; (3) the therapeutic gene, either as cDNA or genomic sequence; and (4) a polyadenylation signal. A wide array of viruses—including murine and human retroviruses, adenoviruses, parvoviruses such as AAV, herpes viruses, poxviruses, toga viruses, and nonviral plasmid therapy systems—have been developed for gene therapy applications. These vectors (see *Table 3*) differ greatly in terms of their capacity to deliver genetic material and the duration of expression. Some viral vectors preferentially target dividing cells, but others are capable of transducing both dividing and nondividing cells. There are significant variations in transgene capacity (i.e., there are limitations on the size of the foreign DNA fragment that can be incorporated into the vector genome). The level, timing, and duration of gene expression required for a gene therapy product depends on the clinical indication. Low-level, long-term gene expression may be required for some diseases, including adenosine deaminase (ADA) deficiency or type A and type B hemophilia. High-level, short-term expression may be more appropriate for cancer when genes that induce apoptosis are used or for cardiovascular disease when preventing hyperproliferation of smooth-muscle cells that may impede restenosis of saphenous vein grafts.

Table 3. Types of Gene Vectors

	Viral							**Nonviral**
Family	**Retroviridae**		**Adenoviridae**	**Parvoviridae**	**Herpesviridae**	**Togaviridae**	**Poxviridae**	
Examples species	**Murine Leukemia Virus**	**HIV**	**Adenovirus**	**AAV**	**Herpes Simplex Virus**	**Sindbis**	**Poxvirus (Vaccinia)**	**Plasmid derived**
Vector Characteristics								
Insert size limit	8 kb	8 kb	4.3–34 kb	4–5 kb	40–150 kb	5 kb	25–50 kb	12 kb
Chromosome integration	Yes	Yes	No, episomal	Can be integrated or episomal	Can be integrated or episomal	No	No	Yes, but at very low frequency
Therapeutic expression	Stable	Stable	Stable or transient	Stable	Stable or transient	Transient	Transient	Stable or transient
Vector localization	Nucleus	Nucleus	Nucleus	Nucleus	Nucleus	Cytoplasm	Cytoplasm	Nucleus
Types of cells transduced	Dividing only	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent
Efficiency of gene transfer	High	High	High	High	High	High	High	Low
Expression of viral proteins	No	No	Yes, unless viral genes deleted	No	Yes	Yes	Yes	No

Table 3. Types of Gene Vectors *(Continued)*

	Viral							Nonviral
Family	**Retroviridae**		**Adenoviridae**	**Parvoviridae**	**Herpesviridae**	**Togaviridae**	**Poxviridae**	
Examples species	**Murine Leukemia Virus**	**HIV**	**Adenovirus**	**AAV**	**Herpes Simplex Virus**	**Sindbis**	**Poxvirus (Vaccinia)**	**Plasmid derived**
Vector Characteristics								
Other	Tropism can be altered by pseudotyping					Can be used as a plasmid therapy system		

VECTOR DESIGN CRITERIA

Many types of gene therapy vectors are being developed, and the vector selected for a particular clinical application depends on the disease state, the target cell, and the intended route of administration. As shown in *Table 3*, capacity depends on vector type, so clinical applications that require a large amount of genetic material will limit the choice of vector system. The payload of a vector system becomes increasingly important when one designs vectors with genomic DNA or a vector that contains extensive regulatory sequences.

Vectors are also selected based on the intended duration of expression and the target cell. For example, retroviral vectors integrate stably into target cells and are therefore well suited for stem cells or lymphocytes that are expected to undergo extensive cell division. In contrast, adenoviral and plasmid vectors are episomal and may be lost during cell division. However, adenoviral vectors are attractive for vaccine development and cancer applications where tumor cell elimination is the goal. Other vectors, such as AAV, do not integrate at high efficiency but can be expressed long-term in nondividing cells such as neurons or hepatocytes.

Target cell type can also play into the selection of an appropriate vector system (see *Targeting Transduction*). For example, the adenoviral Coxsackie virus B and adenovirus receptor (CAR) is expressed poorly on hematopoietic tissues, which limits the usefulness of the vector system for blood-derived cells. Vectors based on murine retroviruses require cell cycling and are not well suited to the transduction of nondividing cells such as neurons.

The immune system can target both the viral components of the vector and the expressed transgene. Pre-existing antibodies or cellular immunity to certain vector systems can exist and may limit their usefulness. Vectors can elicit an innate immune response that can decrease the efficiency of gene transfer and may also induce a severe adverse event. A large number of current gene therapy approaches seek to limit toxicity and immune response by administration of vector to cells ex vivo. Nevertheless, the majority of diseases suitable for gene therapy will require in vivo administration, and ongoing research seeks vectors with limited immune recognition.

The route of administration and manipulation of the total dose of vector are strategies that can be used to compensate for some limitations of specific vector systems. Additionally, there are advantages and disadvantages for the manufacture of each of the different vector systems that should be considered when planning a clinical application. Production consistency favors systems with well-defined fermentation or culture systems, such as plasmid, retroviral, or adenoviral vectors. For viral vector systems that require helper functions (see below), a rationally engineered cell line can overcome the scalability and consistency limitations of co-transfections. Use of a cell line that is adapted to suspension culture can affect scalability and cost efficiency.

TARGETING TRANSDUCTION

To be effective, a vector must first find and transduce its target cell. Viruses have a natural host range that is strongly influenced by the expression of specific cell-surface receptors, the current phase of the cell cycle, and the route of administration. Integrins are a class of cell-adhesion receptors that interact with either the penton base or the fiber protein of adenoviruses. The fiber and penton base proteins of adenoviruses mediate binding to the CAR, CD46, and integrins. Adeno-associated viruses primarily interact with heparan sulfate proteoglycan and sialic acid receptors on the cell surface. However, interaction with secondary receptors such as integrins, laminin, and growth factor receptors is required for efficient cell entry and trafficking of virus particles to the nucleus. An amphotropic variant of the murine leukemia virus (MLV), commonly used for gene therapy applications, utilizes the sodium-dependent phosphate transporter RAM-1 to enter cellular targets. Expression levels of each of these receptors vary according to tissue type, which dictates the transduction efficiency of the vector.

The host and tissue range can be modified or targeted by biochemical and genetic manipulation of the vector. Alterations in the tissue and cell specificity of retroviruses—and lentiviruses in particular—occur largely though genetic pseudotyping. During this process, the envelope proteins that dictate virus binding and entry via a specific cellular receptor of one virus are replaced with the envelope protein of another retrovirus or with a protein from an entirely different virus such as the vesicular stomatitis virus glycoprotein. The relative complexity of adenovirus and adeno-associated virus capsids allows them to be genetically modified in several ways. Substitution of a single virus protein (e.g., adenovirus fiber or AAV VP1) with that of another serotype within the same family is very much like the pseudotyping process for retroviruses. However, mosaic virus particles created by

interspersing individual capsid proteins from several different virus serotypes in one virion, and chimeric particles created by capsid proteins of two distinctly different serotypes on a particle of yet another serotype (e.g., adenovirus 35 knob on adenovirus 41 fiber on an adenovirus 5 particle) can also effectively change the types of cells and tissues that a vector can transduce. Although this approach may seem straightforward, certain modifications of virus capsid proteins at the genetic level do not facilitate virus particle formation. Although most modifications improve virus uptake in one specific cellular target, they may also increase uptake in several other tissues in which gene transfer would not be desirable. Thus, selection of proteins and ligands must be carefully considered and tested in preclinical models of disease before these vectors can be used extensively.

Viral coat proteins and nonviral vectors can be chemically modified for ligand-mediated receptor targeting. They can be conjugated to cell-targeting ligands by antibody–virus interactions with bi-specific antibodies. Molecular bridges like biotin–avidin complexes and chemical crosslinkers such as bifunctional polyethylene glycol (PEG) tethered to cell specific receptor-binding proteins are easily conjugated to virus particles and often are incorporated in targeting strategies. These approaches can easily be combined and/or exchanged with genetic modifications and with each other to create vectors that effectively target several receptors. Although the biochemical approach avoids the functional complications of introducing foreign domains into viral proteins, each lot of vector must be modified because progeny virus will default to their original genetically encoded tropism. Biochemical processes also require the use of multiple reagents, which may complicate transfer to the clinic.

Another common and effective strategy to target viral vectors to tumor cells takes advantage of the virus replication cycle. Deletion of genes critical for taking over functional cellular checkpoints to support normal virus replication allows the virus to replicate only in cancerous cells where those checkpoints are either defective or inactive. This effect can be further enhanced creating small mutations in virus replication genes driven by tumor- and tissue-specific promoters. With respect to cell cycling, adenoviruses and adeno-associated viruses easily infect both quiescent and dividing cells, but MLV-based retroviral vectors are efficient only when transducing rapidly dividing cells. Lentiviral vectors can infect quiescent cells, including cells of neuronal origin. In general, nonviral vectors can enter both dividing and nondividing cells but have lower transduction efficiencies than viral vectors. Transduction efficiencies of nonviral vectors can be enhanced by the formulation and direct injection in the tissue of interest.

General Chapters

INFLUENCE OF HUMORAL IMMUNE SYSTEM AND COMPLEMENT

One of the most significant barriers to effective gene transfer is the humoral immune response to the vector. Regardless of the route of administration, the intended target cell, and the dose, the vector is likely to encounter some component of the immune system. For viral vectors, the humoral immune system cannot readily distinguish between wild-type viral infections and recombinant viral vectors because the humoral response is directed against proteins in the viral envelope or capsid. Protein-containing formulations of nonviral vectors can also elicit a humoral immune response. Specific and cross-reacting humoral responses may pre-exist because of natural exposure to wild-type versions of viral vectors, or they may be elicited during dosing, and the antibody response may vary in its capacity to diminish gene transduction in individual patients. Because neutralizing capacity is frequently enhanced upon multiple dosing, repeated administration can also be problematic. Some of these issues may be remedied by the use of nonviral systems. However, the level of transduction efficiency of these vectors is not currently sufficient for many gene-transfer applications. Transduction efficiency of all vectors is also importantly compromised by the complement system. Many complement proteins have a natural affinity for virus capsid proteins. This interaction initiates release of cytokines and chemokines that facilitate rapid removal of vector from the systemic circulation. This affinity is often heightened (especially in the case of retroviruses) when nonhuman cellular proteins and culture components (e.g., fetal bovine serum) are incorporated into and/or coat the virus particle during large-scale production. Use of producer cell lines of human origin and serum-free culture conditions has decreased inactivation of vectors by complement.

Several strategies for mitigating and avoiding the humoral immune response and complement inactivation have been developed. Increasing the vector dose to compensate for the neutralizing activity of the antibodies and altering dosing regimens to coincide with periods of low antibody titer are logical choices, but possible toxicity associated with high doses of virus and the individual variability of the immune response are potential negative consequences of this approach. Alternatively, viral vectors can be engineered to evade the immune system. One approach involves increasing expression of specific viral genes that allow the virus to evade the host's humoral response. Recombinant viruses constructed from serotypes with limited exposure rates such as simian adenovirus 7 can avoid neutralization in those previously exposed to more common serotypes. Mosaic and chimeric viruses can also avoid neutralization. Both approaches effectively address the issue of efficient gene transfer in those with pre-existing immunity, but both approaches require further investigation in response to concerns regarding safety and large-scale production as well as induction of immune responses in naive patient populations. Covalent attachment of polyethylene glycol (PEGylation) to the virus capsid can both protect the virus from neutralization and blunt the immune response. Pharmaceutical methods such as embedding viral vectors in polymer matrices and administration of vectors to the mucosa (oral and nasal) can also protect viral vectors from the humoral immune response.

INFLUENCE OF CELLULAR IMMUNE RESPONSES

Once transgene expression is initiated, cellular immune responses rapidly remove cells transduced by both viral and nonviral vectors. This decreases the overall therapeutic effectiveness of gene transfer of low-to-moderate vector doses and can be highly toxic when higher doses are administered. Active protein synthesis is not required for cellular immune responses to viral capsid

proteins. For example, the capsid proteins of recombinant AAV vectors have been shown to be long-lived, leading to a delayed immune response and elimination of vector-transduced cells. De novo synthesis of viral gene products can also exacerbate host-cellular responses. Viral vectors have been designed with specific backbone deletions to eliminate the expression of viral structural genes and reduce this effect. Examples of such vectors include gutless adenoviruses, herpes viruses, and adeno-associated viruses in which all viral genes have been deleted, making them dependent on another helper virus for subsequent replication. Certain plasmid sequences, especially those with unmethylated CpG dinucleotides, can elicit a strong cellular immune response and have been used as adjuvants in some DNA-based vaccines. Amplifying plasmids in bacteria that express the CpG Methylase (M.SssI), removal of CpG sequences by site-directed mutagenesis, and removal of unnecessary prokaryotic sequences to create minimal plasmids have reduced the incidence of unwanted cellular responses. One nonmolecular approach to minimize the cellular response against the vector involves the use of immunosuppressants (cyclosporine, sirolimus, daclizumab) at the time of initial vector administration as well as methods described for reducing the humoral response (use of chimeric vectors, PEGylation) outlined above.

ANTIGENICITY OF THE GENE THERAPY PRODUCT

In many cases, the gene therapy product and associated promoter and enhancer elements are antigenic in certain cellular targets. When proteins that are retained in the target cell are used, cellular responses may eliminate the target cell. If sustained protein expression is required, the cellular immune response may decrease the effectiveness of the therapy or eliminate it entirely. In terms of treating genetic diseases, patients with a null mutation who have never seen the transgene product may be at a higher risk for immune response than patients who produce a defective protein. Also, truncation of a gene such as the cystic fibrosis transmembrane conductance regulator (CFTR) so that it fits within a chosen vector may result in creation of a distinct antigen.

In other applications, the transgene product is a foreign protein, e.g., thymidine kinase derived from the herpes simplex virus (HSV), and thus may elicit an immune response. In some cases this is the desired therapeutic effect, particularly in antigen-based immunotherapy for cancer or a viral disease. Efforts to minimize the immune response against elements associated with the transgene cassette include designing vectors with the ability to carry full-length humanized sequences for the transgene of interest and administration of immunosuppressants at initial dosing and other intervals throughout the treatment protocol.

VECTOR LOCALIZATION WITHIN THE TARGET CELL

Once the vector reaches the target cell, several factors can affect the level and duration of therapeutic gene expression, and these factors dictate the choice of an appropriate vector system for a specific clinical indication. The localization of the vector genome within the cell, the strength of the gene expression control elements, the stability of the message, and the stability of the translated protein all affect therapeutic impact. Alphavirus-based vectors, such as those derived from Sindbis or Semliki Forest virus, reside in the cytoplasm and typically exhibit a very high level of gene expression. Retroviral, adenoviral, and other viral vectors have advantages in gene delivery with their natural mechanisms for nuclear delivery of the therapeutic gene and reasonable levels of gene expression from viral or other promoters. Nonviral plasmid vectors are episomal and are often susceptible to DNA degradation when they are shunted into cell endosomes. However, some nonviral systems incorporate nuclear targeting signals as a means of increasing therapeutic gene transcription efficiency.

TISSUE-SPECIFIC EXPRESSION

Another means of controlling gene expression is the incorporation of tissue-specific promoters to stimulate or to restrict expression of the therapeutic gene. Unfortunately, many tissue-specific promoters do not provide high levels of gene expression, and incorporating these sequences into viral vectors may result in loss of specificity or low-level expression in cells that do not normally express the promoter. Tagging vector with sequences recognized by the microRNA system has also permitted tissue-specific expression and may offer tighter control than typically seen with tissue-specific promoter systems.

Drug-responsive promoters are being used to control gene expression. Rapamycin, mifepristone, and tetracycline (tet-on) systems have been used to repress gene expression. This type of regulation is particularly useful when constitutive expression of the vector transgene is toxic.

IMPACT OF REPLICATION STATUS OF VECTOR

Replication status is another important consideration for vector design and selection. Viral vectors are most frequently constructed to be incompetent or replication-defective in order to limit uncontrolled vector spread and pathogenicity. However, the ability to replicate and spread within a specific cell population, e.g., within a tumor or to metastatic sites, may provide a significant therapeutic advantage over cell-type–specific targeting of replication-incompetent vectors. Replication can be engineered to be conditional when, for example, specific viral gene interactions are matched with intracellular pathway targets by means of targeted deletions and/or changes in transcriptional or translational control. When these targets are defective or missing, as in cancer cells, the virus can replicate, but when the target cell is functioning normally, viral replication is repressed.

Viruses that have been genetically engineered for selective oncolytic replication include: adenovirus, HSV, vaccinia, measles virus, picornaviruses, influenza virus, Coxsackie virus, and Sendai virus. Some nongenetically-modified viruses are inherently oncolytic in human cells, e.g., reovirus and Newcastle disease virus.

One of the risks inherent in the use of conditionally replicating viral vectors is that such systems are leaky, i.e., the growth of the virus is not absolutely restricted to a single cell type. Also, subsequent rounds of viremia become considerations in the evaluation of tissue distribution/exposure and shedding. The therapeutic promise of these approaches depends on the reliability with which conditionality of replication can be selected or engineered. This therapeutic potential will be realized only if balanced with steps to control the potential risks to patients (associated with replication competence of the viruses/viral vectors) and to address associated shedding-related issues of third party exposure and environmental concerns.

As a proactive contribution to the safety profile and to take advantage of scientific and clinical information already available, virus strains that have been used for human vaccination are often used as the vector backbone. Nevertheless, because the product is replication competent, it presents specific technical challenges for adventitious agent testing and product characterization.

Nonviral vectors are normally designed as nonreplicating systems, but some groups are experimenting with replicating nonviral plasmids to increase gene expression levels (because of the low transduction efficiency of most nonviral systems) and to increase the duration of gene expression. Additional preclinical studies are needed to establish the safety of these systems. Artificial chromosomes have also been designed to take advantage of normal mechanisms for retaining gene expression in rapidly dividing target cells.

VECTOR INTEGRATION

The duration of gene expression is also a function of the persistence of the vector genome in target cells. Retroviral vectors can stably integrate into the host-cell genome, providing long-term expression. Adenoviruses and nonviral plasmid vectors, e.g., those not administered using electroporation, do not integrate, and expression generally decreases over time. Recombinant AAV vectors generally do not integrate, and when they do, it is not site specific. However, stable episomes have been observed in certain cell types such as muscle cells.

Site-specific integration can be a desirable feature for vectors that are intended to correct genetic disorders. Although it is not currently efficient enough to be useful, the control of the site of integration is desirable in order to prevent insertional mutagenesis. Insertional mutagenesis has the potential to kill a cell if a critically functioning gene is inactivated or to predispose a cell to malignant transformation if a tumor-suppressor gene is inactivated. Of clinical relevance, promoter or enhancer elements within vectors can lead to activation of cellular oncogenes and have been associated with malignant transformation in children undergoing retroviral gene transfer for X-linked severe combined immunodeficiency.

The success of any gene therapy product depends on the relationship between the vector-delivery system and the requirements of the disease in terms of the site, level, and duration of therapeutic gene expression. A universal vector now appears unlikely, and the challenge lies in fitting one of several possible vectors to the disease and to the gene to be delivered.

Manufacturing and Purification Strategies

VECTOR CONSTRUCTION

Viral and nonviral gene-transfer vectors are constructed by using standard molecular biology protocols. For viral vectors, the vector backbone consists of viral RNA or DNA sequences from which the regions encoding viral structural genes or the regions required for replication have been deleted. The deleted region of the vector is usually modified with specific restriction endonuclease sites used to allow insertion of the gene of interest. For nonviral vectors, the plasmid DNA backbone contains multiple restriction sites for cloning and the bacterial elements necessary for plasmid production. Vector backbones can accommodate single or multiple gene insertions depending on the maximum amount of sequence they can carry. The promoter that facilitates transcription of the gene insert can be a related viral promoter, such as the murine leukemia virus long terminal repeat (MuLV LTR), or a heterologous promoter that is either tissue specific, such as the alpha crystalline promoter (of the eye), or constitutive, such as the cytomegalovirus (CMV) late gene promoter. For example, in a retroviral vector construct containing two gene inserts, transcription of one is regulated from the 5′-LTR-promoter sequence, and a second gene insert can be linked to an internal heterologous promoter from Simian virus 40 (SV40).

The complementary DNA (cDNA) containing the therapeutic gene of interest, including its introns, is excised from its source using restriction enzymes and is inserted at the multiple cloning site of the gene-transfer vector. The polyadenylation signal can be derived from multiple sources such as the SV40 virus or human growth hormone gene. Characterization and testing of gene therapy vectors are described under *Analytical Methods*.

HELPER FUNCTION SYSTEMS

Recombinant viral vectors are most often modified to be replication defective, a condition created by deletion or modification of the viral genes needed for replication and production of infectious virus. Because the vectors are stripped of some or all

of the viral genes, a system must be developed to supply viral proteins and to encapsulate the vector into a viral particle. Generally, this is accomplished by two methods: transient transfection or stable packaging cell lines.

In the transient transfection method, a series of different plasmids are generated, including a plasmid containing the vector and another vector containing the viral genes. For example, retroviral vectors can be generated using a three-plasmid system: (1) the transgene-containing vector plasmid; (2) a plasmid containing the *gag/pol* viral gene region; and (3) a plasmid containing the viral envelope. All three plasmids are transfected into cells, e.g., HEK293 or HT1080, and vector-containing virions are harvested after two to three days. The separation of vector and viral genes on different plasmids, along with vector designs that minimize the homology between vector and viral sequences, decrease the chance for recombination and generation of replication-competent virus. Similar approaches can be taken with most viral vector systems.

The transient transfection method has the advantage of a rapid production time and flexibility when changing components of the vector or viral constructs. Nevertheless, it can be cumbersome when scaling up for manufacturing, and special care must be taken to provide consistent production yields. An alternative method has been the use of vector packaging cell lines. In this scenario, the viral genes are introduced stably into an immortalized cell line that yields persistent expression of viral genes. As with transient transfection, the viral genes generally are expressed from different plasmids to decrease the risk of recombination. Since plasmids integrate infrequently, considerable time and effort are required to isolate a high-titer packaging cell line and to generate an MCB. Vector constructs can be introduced into cells from the MCB, and researchers, by screening for a high-titer clone may allow isolation of a stable cell line that generates the vector of interest. These cell lines generally can be expanded to great numbers and often produce vector for up to a week at a time, facilitating vector scale-up and product consistency.

Typical helper function systems are as follows:

Retroviral Vector Systems—Initial packaging cells were based on the murine fibroblast cell line NIH 3T3. The PG13 cell line (expressing the Gibbon Ape Leukemia Virus envelope) has been used extensively with a low incidence of recombination events leading to RCV. More recently, the human HEK293 and HT1080 cell lines have been modified to serve as packaging cell lines for retroviruses. The use of a human cell line decreases elimination of vector particles by the human complement system (although this is generally not a concern for vectors used in ex vivo protocols).

Adenoviral Vector Systems—HEK293 cells are widely used to supply the E1 function necessary for efficient adenoviral replication that is deleted from first-generation adenoviral vectors. Other complementing cell lines, such as E1-modified A549 cells (human lung carcinoma) and the PER.C6 cell line (human embryonic retinoblast), have also been created to supply E1 or other missing functions. PER.C6 contains the E1 region under the control of a phosphoglycerate kinase (PGK) promoter and has no flanking adenoviral sequences in order to eliminate production of replication-competent adenovirus (RCA).

AAV Vector Systems—These systems classically use adenovirus-infected HEK293 cell lines transiently transfected with AAV helper plasmid containing the *rep* and *cap* genes, which are required for AAV replication and capsid formation, respectively, and are deleted from the AAV vector. In some AAV production systems, wild-type adenovirus has been removed from the process by using triple transfection of plasmids expressing *Ad* early genes, *rep* and *cap*, and the vector transgene. The HeLa cell line (from human uterine cervical carcinoma) has also been used as a transient production system. More recently, both of these cell lines have been used to establish stably transfected packaging cell lines that express *rep* and *cap* genes and in some cases express the adenoviral functions needed for AAV replication when *rep* and *cap* are present (E1a, E1b, E2a, E4, and VA RNA). AAV production systems using recombinant HSV and Baculovirus have also been developed.

Gutless Adenoviruses—Early manufacturing systems for the adenovirus vector known as gutless adenovirus were similar to classical AAV vector manufacturing systems because HEK293 cells were transiently transfected with helper plasmid containing required adenoviral functions. Development of helper viruses housing a packaging signal flanked by loxP sites and complementing HEK293 cell lines that express the bacteriophage P1 site-specific Cre recombinase has greatly improved the yield of the gutless virus. This technology notably reduces the amount of helper virus contamination by preventing packaging of the helper virus genome while permitting it to replicate and support replication and encapsidation of the gutless vector.

VIRAL GENE THERAPY VECTORS

Retrovirus and adenovirus vectors typically have been produced at laboratory, non-GMP scale by use of traditional cultivation methods for anchorage- and serum-dependent cell lines employing flasks, trays, and roller bottles. Initially, gene therapy vectors were produced by these methods because large volumes of product were not required for early clinical studies. Cell-bank systems are used as the source of cells, and virus banks are the source of virus for clinical production. In many cases, supernatant is collected, clarified, and stored frozen in bags at −70°. In many early clinical trials, unpurified supernatant has been used for ex vivo gene transfer.

Larger-scale upstream production methods have been reported and are commonly used. They include suspension, bioreactor, and fixed-bed or microcarrier culture methods. Some groups have reported adapting their process cells to serum-free culture conditions. Cells are harvested and lysed or supernatant is collected. The harvest is clarified and purified to remove host-cell debris, host-cell DNA, and other process-derived contaminants.

Traditionally, viruses are purified by gradient ultracentrifugation, but this is time-consuming and unsuitable for larger-scale production purposes. The selection of downstream process steps and their sequence is determined by the nature of the virus itself and the upstream process used for manufacturing the virus. As processes are being developed for the manufacture of gene therapy vectors, many different purification steps have been reported. These include ion-exchange and sulfonated-cellu-

lose chromatography, zinc ion affinity chromatography, and size-exclusion chromatography. Typically, DNase or other nuclease treatments are used in the process in order to reduce host-cell or plasmid DNA. AAV production and lentiviral production are complicated by a need for transient transfection or co-transfection of plasmid or helper virus. These processes have generally required anchorage-dependent cell lines that are difficult to scale up. The development of stably transfected cell lines would allow large-scale production.

PURIFICATION METHODS: VIRAL VECTORS

Retroviruses—To date, purification of retrovirus preparations for phase I clinical trials has often been minimal at best, i.e., the simple concentration of culture supernatants is insufficient to meet the stringent quality standards required for in vivo therapy. Centrifugation and microfiltration techniques are very useful for clarification of culture supernatants and removal of cellular debris. Ion-exchange, size-exclusion, and affinity chromatography techniques have also been employed to remove excess salt, serum, and low molecular weight contaminants also concentrated with the virus.

Adenoviruses—Recombinant adenoviral vectors were often purified by cesium chloride density gradient ultracentrifugation. This is still used for research-scale preparations, but the procedure is neither scalable nor efficient for large quantities of clinical-grade virus. The most recent scalable purification methods use anion-exchange chromatography because of the strong affinity of intact virus particles for the resin with respect to that of cellular and individual capsid proteins. Published loading estimates for anion-exchange resins range from 0.5×10^{12} to 5×10^{12} virus particles/mL of resin or 0.14 mg of virus to 1.4 mg of virus/mL resin. Gel filtration and immobilized metal affinity chromatography are often used in polishing steps following anion-exchange purification of recombinant adenovirus-based products.

Adeno-Associated Viruses—The toxicity of cesium chloride, the aggregation of AAV particles, and the fact that adenovirus is not completely removed after extensive centrifugation complicate AAV purification by cesium chloride density gradient ultracentrifugation. Another density separation medium, iodixanol, which is less toxic than cesium chloride and prevents AAV aggregation, has been employed in a single centrifugation step. Passage of the AAV fraction over an affinity column consisting of either a heparinized support matrix or monoclonal antibodies produced against AAV2 strongly increased purity and infectivity of final preparations. These methods are appropriate only for specific AAV serotypes. Ion-exchange chromatography is the most powerful and versatile method for AAV purification, although buffer pH, detergent concentration, and column medium must be tailored for each AAV serotype. Infectivity and purity of preparations obtained from these purification strategies are comparable to those obtained from affinity chromatographic methods and are complete within three hours.

PLASMID OR NONVIRAL VECTORS

Plasmids are double-stranded, circular DNA molecules that exist in bacteria as extrachromosomal, self-replicating molecules. They have been modified to serve as cloning systems, to contain multiple restriction endonuclease recognition sites for insertion of the cloned transgene, and to contain selectable genetic markers for identification of cells that carry the recombinant vector. Plasmid-based, nonviral vectors are frequently used as gene delivery systems for both in vivo and ex vivo gene therapies. These vectors are in the form of naked DNA or are complexed with lipids or other agents that facilitate transfer across the cell membrane and delivery to the cell nucleus without degradation. An advantage of a plasmid-vector system is the efficient production of large quantities of the vector that is easily characterized and avoids the risk of RCV associated with many viral systems.

Nonviral vectors are typically produced by using an *Escherichia coli* bacterial system. Plasmids are transfected into *E. coli*, and an appropriate single bacterial colony is selected and expanded to create an MCB. After reselection of a colony from a bacterial plate inoculated from the MCB, plasmid DNA is isolated from cultures that can range in size from 1 L on a laboratory scale to hundreds of L in bacterial fermenters. Plasmid DNA can be purified by several methods including affinity or ion-exchange chromatography and cesium chloride–ethidium bromide density gradients. Cesium chloride–ethidium bromide density gradients are not recommended for production of clinical-grade material.

PRODUCTION AND PROCESSING OF NONVIRAL VECTORS

One benefit of nonviral vectors for gene transfer is that the production process is rather generic and can be applied to any plasmid preparation regardless of composition or application. Because the current average human dose of plasmid DNA for gene transfer and vaccination is approximately 1 mg, the primary challenge associated with large-scale production of plasmids is to develop a process that is both scalable and economical. Thus, process development for plasmid-based vectors remains an active area of research and development. A standard process for large-scale production of recombinant DNA plasmids consists of the followinfg five unit operations.

Fermentation—Fermentation processes must support growth of transformed bacteria and maximize the amount of plasmid produced by each cell. *E. coli* is the most common strain used for plasmid production. Amino acids, nucleosides, and the ratio of nitrogen to carbon-containing compounds present in a rich media formulation greatly improve plasmid yield.

Harvest—Bacterial cells are harvested either by centrifugation or microfiltration. Centrifugation under GMP conditions can be costly, which makes microfiltration the accepted method of cell harvest. This also allows spent media, metabolic byproducts, extracellular debris, and impurities to be washed away before purification.

Lysis—Bacterial cells must be lysed to release the recombinant plasmids. This is one of the most critical steps in the production process because it can significantly affect the amount of usable [covalently closed circular (ccc)] and unusable [sheared, partially denatured, and open circular (oc)] forms of DNA in a preparation. The most widely used method of lysis for clinical-scale manufacturing is treatment with alkaline detergent and precipitation of cellular debris with acetate. This removes a large fraction of cellular impurities from the lysate, but it also increases the sensitivity of plasmids to mixing and localized concentrations of detergent, which are hard to manipulate on a large scale. Lysis of cells by heat exposure addresses this issue and effectively denatures cellular proteins and bacterial DNA.

Isolation/Purification—Some processes include additional steps for removal of cellular debris and other contaminants from crude bacterial lysates by precipitation with detergents, polyethylene glycol, or salt. These reagents affect plasmid stability and are removed by column chromatography. Size-exclusion chromatography can effectively separate plasmid DNA from RNA, proteins, and other small molecules present in the cleared lysate. The degree of separation of plasmid DNA from contaminants is highly dependent on the type and concentration of salt in the running buffer. Resins used in anion-exchange chromatography have a high affinity for plasmid DNA and provide maximal sample concentration. Hydrophobic interaction and thiophilic aromatic chromatography are the methods of choice for selective separation of the different plasmid DNA isoforms and endotoxin reduction.

Bulk Preparation—After purification, the bulk plasmid is placed in a suitable buffer and formulation by ultrafiltration using a membrane with a pore size of 50–100 kDa.

Plasmids for clinical use must be highly characterized. Impurities from production and processing steps are well known. Tests necessary to confirm the identity, purity, and potency of a plasmid-based product are well established and routine. These tests and the current specifications set by FDA and the World Health Organization are summarized in *Table 4*.

Table 4

Assay Type	Issue	Determined By	Acceptable Level in Final Product
Identity	Cross-contamination with other products	Restriction digest/gel electrophoresis	N/A
	Residual bacterial chromosomal DNA	Real-time PCR	<2 µg/mg DNA
	Residual RNA	Analytical HPLC	<0.2 µg/mg DNA
	Residual bacterial protein	BCA protein assay	<3 µg/mg DNA
	Endotoxin	LAL assay	<10 EU/mg DNA
	Sterility (bacterial and fungal)	Method outlined in 21 CFR 610.12	No growth
	Appearance	Visual inspection	Clear solution free of particulates
	pH	pH meter	Physiologic (7.0–7.4) but may be product specific
Purity	Plasmid confirmation (ccc vs oc)	HPLC or CGE	>97% ccc
Potency	Labeled dose	In vitro ELISA FACS RT-PCR Light absorbance (A_{260})	Transgene/plasmid specific

Introduction of Genetic Material into Cells—Gene-Modified Cells

A common extension of cell therapy involves the introduction of genetic material, usually DNA, into cells to alter their pattern of gene expression. While discussion focuses on DNA, similar scenarios can be applied to RNA or a derivative of DNA, except that the stability and solubility of the particular nucleic acid may dictate modifications of certain steps. The general process is often referred to as ex vivo gene therapy because the cells are removed from the patient or donor and the genetic material is introduced while the cells are outside of the body. The genetically modified cells are then administered to the patient. The genetic material introduced can either cause expression of new genes and products or inhibit the expression of already expressed genes and products. The latter represents a type of antisense therapy. The genetic material can be introduced by the same range of reagents that are involved with gene therapy: viral vectors, nucleic acids in a simple formulation (naked DNA), or nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Most of the steps and considerations discussed above also apply to the ex vivo introduction of genetic material into cells. The main goal of ex vivo therapy is to develop robust processes that will work with the majority of the patient's or donor's cells. This takes considerably more effort than processes for cell lines.

The method for introducing new genetic material into cells depends on the biology of the system and the desired stability of gene expression. If a simple retroviral vector such as Molony murine leukemia virus is used for transduction, the cells must be actively dividing because vector DNA is integrated into the cellular DNA only during replication. This usually leads to long-lasting expression of the desired gene product. Adenoviral vectors, naked DNA, or formulated DNA can be introduced into nondividing cells. However, gene expression will be transient because the introduced DNA will usually be extrachromosomal.

The main challenge in ex vivo gene therapy is to achieve efficient transduction or transfection, introducing sufficient DNA into the cell before the DNA degrades. In the case of transduction by simple retroviral vectors, cells are stimulated with reagents that cycle them into the S phase (replication) at the time that the vector is applied. Most retroviral vectors are stable in cell culture for a period up to a few hours. Because diffusion is minimal, only a small fraction of viral particles will come into contact with cells during this period. The following techniques can be used to increase the number of viral particles that contact the cell in a given time period:

1. Maximization of viral particle concentration and minimization of the media volume during the transduction step
2. Multiple applications of the virus
3. Centrifugation of virus particles onto the cells
4. Placing cells on a filter and slow pulling viral media through the filter
5. Addition of binding-enhancing polymers to the media. [NOTE—Co-culturing the target cells with the viral producer cells is not recommended. This technique increases the chance of a recombinant event and production of RCV. Furthermore, any product for which co-culturing is used to transduce the human cells would be considered a xenotransplant if the producer cells were not human. The second cell type, whether human or not, may cause inflammation.]

Each of these techniques has its own set of issues that must be addressed in order to develop a robust process. In technique 1, reduction of the volume during transduction results in rapid exhaustion of the medium, so supplemental medium should be added within a few hours. In technique 2, the cells may no longer be in the correct cell cycle phase during later applications, or cells may have become refractory because of unproductive transformation during the previous application. Techniques 3 and 4 can work well on a very small scale, but the number of cells that can be transduced may be insufficient to obtain an efficacious dose. In technique 5, polymers may fail to provide a benefit because virus binding may involve specific receptors for which surface density may prove to be the limiting factor.

Similar issues and techniques can apply with other viruses or DNA preparations. The issue of slow diffusion is even more marked for the use of DNA preparations. Factors such as the cell type in which the viral vector was produced, the media used for vector production, and the purity of the vector can have a pronounced effect on the efficiency of transduction. Although certain methods may not require cells to be actively cycling, in practice, most processes require that cells be capable of replication because of the following considerations:

1. Safety considerations may dictate that only cells that express the new DNA are returned to the patient, which requires that these cells be selected. As described below, the most common selection method uses an antibiotic-resistant gene that is co-introduced with the new genetic material.
2. Further propagation may be required to achieve the therapeutic dose of cells.
3. Economic, biological, or technical reasons may dictate that the DNA introduction step be carried out at a low cell number and that the desired cell population then be expanded to the required dose.

Therefore, conditions that enable the cell or maintain its ability to proliferate must be developed in almost all cases. The biology of the cells, the available technology, and process economics will determine whether cells are propagated before, after, or during the introduction of new genetic material. Most processes do in fact expand the population after the introduction of the new gene.

Whether cells that do not productively express the gene can be administered to patients depends on the biology of the application, the dose required versus the handling capability of the manufacturing system, and, most importantly, the toxicity of the nonproductive cell population. Selection of the genetically modified cell population is commonly carried out using an antibiotic-resistance marker gene, such as neomycin, which is co-introduced into the cell with the new genetic material. For neomycin selection, cells in culture are treated with the antibiotic G418 at a concentration and for a period that has been shown to kill cells with nonproductive expression while allowing the productively expressing cells to proliferate. In this manner cells that are resistant to the antibiotic are presumed also to express the DNA of interest. The expression should be tested as a lot-release requirement or verified in a series of mock runs. Because most antibiotics decrease cellular proliferation, optimization of the culture media composition may be necessary for efficient selection and propagation of the gene-modified cells.

Following the antibiotic selection step, a second phase of antibiotic-free cell propagation may be required in order to achieve the desired dose and to rinse residual G418 out of the system. The selected medium and the total time that the cells are in culture can be critical to maintaining the desired expression of the original differentiated functions. An additional issue associated with the use of selection markers is that they generally are nonhuman genes. The expression of these genes usually elicits an immune response. Process development is often carried out with cells from healthy donors. Consideration should be given to the fact that for very sick patients, healthy cells that can be stimulated to undergo efficient, sustained replication are difficult to obtain.

Manufacturing, cell processing, and analytical testing issues pertinent to cell-based products are addressed in chapter ⟨1046⟩.

General Chapters

Formulation of Gene Therapy Products

Final formulations for gene therapy products are still in early development, and currently most gene transfer vectors are stored in solution at ultra-low temperatures. Successful formulation of candidates for gene transfer relies on a thorough understanding of the physicochemical and biological characteristics unique to each vector system. Factors like solution pH, ionic strength, and osmolarity influence the thermal stability of viral and nonviral vectors. Organic carbohydrates such as mannitol, sorbitol, sucrose, and trehalose have been incorporated into preparations to prevent disruption of the native conformation of the vector in solution, during the freeze-thaw process, and during lyophilization. Amino acids such as arginine and leucine have been incorporated into formulations for their buffering effects and to prevent aggregation. Surfactants such as the Tweens, Spans, and Pluronics have been effective at preventing aggregation, but this effect is somewhat vector specific because some vector products are easily disrupted by these reagents. Lipids, polymer and extraneous proteins (human albumin and gelatin) have also been incorporated in many vector preparations because of their ability to prevent loss of vector from direct interaction with pharmaceutical surfaces and during freeze-thaw cycles.

Before initiating a program for formal screening of formulations for a vector, the following factors should be considered. The required dose and/or storage concentration of the final product, as well as the specifics of the container-closure and/or delivery system should be established. Analytical methods to assess potency and identify degradants should be in place. The expectations of the formulation must also be defined. Some pragmatic criteria for the design and selection of vaccine formulations for use worldwide are: (a) the final product should be in a formulation that affords an 18- to 36-month shelf life when stored at 2°–8° or above, (b) the formulation should have an acceptable stability profile at ambient temperatures to cover short-term storage and transportation in the field, (c) it should adequately protect the vector from damage during freeze-thaw cycling, and (d) it should consist of reagents that are pharmaceutically acceptable and within physiologically acceptable concentrations. Formulation changes during clinical development must be supported with preclinical studies and stability data. Sufficient time should be built into plans to account for this.

To date, little work in the area of formulation development of retroviral vectors with additives approved for human use has been described. The most significant effort to develop stable formulations for gene transfer has been with recombinant adenoviruses. Recently, identification of the mechanisms by which recombinant adenoviruses degrade in solution led to the development of several liquid formulations that stabilize the virus for up to 18 months at 4°. Adeno-associated viruses are regarded as one of the most stable viral vectors. This virus has been documented to be stable for approximately 4 months in phosphate buffered saline at 4°. Addition of cryoprotectants and surfactants prevents aggregation of virus particles and extends the shelf life to one year. Lyophilized formulations of both adenovirus and AAV with shelf lives of several years at room temperature have also been described. Although nonviral vectors have been found to be generally robust in standard buffers at 4°, their stability may be extensively influenced by extraneous components included to promote gene targeting. Consider the nature of those components when developing stability protocols and strategies (see below).

ON-SITE PREPARATION AND ADMINISTRATION

One or more product modifications or preparative steps may be required before administration of the gene therapy product to the patient. These modifications or steps are frequently performed close to the time of administration, and, therefore, they are performed under conditions not under control of the original manufacturing facility. The nature of these modifications is dictated largely by characteristics of the product in relationship to the particular application. These include thawing, washing, or filtration to remove unwanted product manufacture-related materials and also include defined physical space with appropriate environmental controls, trained personnel, detailed standard operating procedures, and a comprehensive quality program.

The unique and irreplaceable nature of many gene therapy products, e.g., gene-modified cells, many of which have originated from an autologous or a selected allogeneic tissue source, creates special considerations for product manufacture, release, and administration. Issues pertinent to the administration of cell-based products are addressed in detail in chapter 〈1046〉.

On-Site Preparation

PRODUCT MANIPULATIONS

Before administration, on-site preparation of the gene therapy product may involve one or more manipulations, including the following:

- **Change in final container**—The manufactured product may have been stored or transported in one container but may require transfer to a different container for administration.
- **Change in physical state or temperature**—A product may require thawing from the frozen state or warming from the refrigerated state.
- **Change in solution or suspension**—A product may have to be dissolved, diluted, or suspended in a liquid.
- **Addition to biocompatible structural material**—A gene therapy product may need to be combined with living, natural, or synthetic structural tissue or matrix. Examples of matrix material include hollow fibers, fibrous sheets, gels, plugs, capsules, sponges, or granules.

General Chapters

- **Admixture or compounding with other nonstructural materials**—A product may require mixing or compounding with drugs, cytokines, biologics, or other nonstructural materials.
- **Filtration or washing**—Unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations may require washing or filtration steps.
- **Sampling**—Sampling of the final product immediately before administration may be required for certain clinical protocols.

FACILITY REQUIREMENTS

Facility requirements for performing on-site preparative steps or administration of gene therapy products depend on the nature of the products, their applications, and the manipulations required. The most important determinant of facility features is the level of risk for microbial contamination associated with each step. Definition of low-risk and high-risk conditions can be made according to a framework similar to that defined for Low-Risk-Level Compounded Sterile Preparations (CSPs) and High-Risk-Level CSPs in *Pharmaceutical Compounding—Sterile Preparations* 〈797〉, *CSP Microbial Contamination Risk Levels*.

RELEASE OF FINAL PRODUCT

Gene therapy products that undergo on-site preparative steps or manipulations must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations will determine whether release requirements or critical specifications must be added to those required immediately after initial manufacture. Pre-release requirements usually include the following:

1. Physical inspection of the product, which typically includes measures to ensure appropriate product appearance with regard to color, turbidity, particulates or foreign matter, container integrity; product temperature; and accuracy and convenience of labeling
2. Review of process records
3. For patient-specific products, clerical checking of product labeling or records related to the identity of the intended recipient.

In addition, products considered to be high-risk products according to the description in chapter 〈797〉 should undergo additional product testing. For all high-risk products, quality assays for the identity, potency, and purity of the active ingredients should be defined and performed. For high-risk products in Category II, sterility and endotoxin testing should be performed.

Administration to Patients

PRE-ADMINISTRATION REQUIREMENTS

Depending on the specific gene therapy application, trained patient-care staff must take steps to prepare the patient for product administration. These steps are aimed at ensuring that the product will provide the intended therapeutic outcome and at minimizing the risk of adverse effects. Issues pertinent to administration of cell-based products are addressed in chapter 〈1046〉. Generally, a thorough re-evaluation of the patient's general condition and suitability for therapy must be performed close to the time of product administration. This evaluation usually includes a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, staff may obtain baseline physical or functional measurements, laboratory tests, or imaging studies relevant to the specific application. Examples include pulmonary function tests for a therapy aimed at improving lung function, measurement of blood levels of an enzyme that is the gene product in a gene therapy application, and nuclear imaging of organ before anticancer therapies.

A variety of patient interventions related to route of administration may be required before product administration. For therapies that require intravenous administration, patients with poor peripheral venous access may require placement of a central venous catheter. In applications where gene-modified cells or matrices combined with cells are implanted into the patient, the site of implantation may require preparation in the operating room. This may involve surgically opening the site, removing the degenerated or damaged tissue, trimming of the adjacent tissue to accommodate the implant, and excising the tissue from a second site to be used as an anchor or support for the implant. For instance, in the case of products for wound healing, it is critical that the site for grafting be free from infection and that it demonstrates a well-prepared wound bed. Where gene-modified cells are intended to repair cartilage defects, the site of damage needs to be prepared so that the cells can be applied to a water-tight compartment. For applications involving direct administration of the product into an organ system (for example, bronchioalveolar system) or vascular network (for example, coronary arteries), the patient may require endoscopic or surgical access to these sites.

In all cases, the need for adequate anesthesia and premedication must be carefully evaluated in conjunction with these steps before product administration. Pre-administration patient evaluation must also include assessment of concurrent therapies that may interact with the gene therapy product to modify its effects. Some therapies may be considered adjunctive to the gene therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly

used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects on the efficacy of the gene therapy product.

PATIENT TREATMENT

Some gene therapy products are patient-specific because they are manufactured from a selected tissue source, such as autologous, selected allogeneic, or xenogeneic tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, including special attention to the correct identification of the patient and patient-specific product by at least two people immediately before administration. These issues are addressed in greater detail in ⟨1046⟩. Gene therapy products can be administered by a variety of routes. These include parenteral injection, inhalation, and gastrointestinal routes. Other possibilities include direct application of gene therapy products into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. Although parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities such as an aseptic operating theater or endoscopic suite. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

POST-ADMINISTRATION MONITORING OF PATIENT

There should be written policies and procedures for monitoring patient outcomes and managing reports of adverse events. Patient outcome assessment should include indicators that are likely to detect errors or problems related to the entire manufacturing process, with special attention to manipulations, storage, or transportation after the initial manufacture of the product. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients with suspected adverse effects and a system for reporting and evaluating adverse effects that may point to a potential defect in the administered product. Reporting procedures include providing details required for federal, state, or USP adverse-event reporting programs.

Follow-up and monitoring procedures should be implemented for patients who have received gene therapy vectors or ex vivo gene therapies. To the extent that it is relevant and that it can be assessed, vector or gene-modified cell biodistribution and persistence in vivo should be monitored. With direct administration of vectors, localization to the germ line may be an issue. Although preclinical studies can address this issue, useful information may be gained by patient monitoring. When a retroviral vector has been administered, patients should be monitored for replication-competent retrovirus (RCR) according to FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors* (October 2000). This involves active monitoring during the first year and archiving of patient samples thereafter if RCR is not detected initially.

Database systems to collate and track patient-monitoring results are essential to management of this information. National registries or publication of data should be considered for establishing the collective safety of gene therapy.

ANALYTICAL METHODS

The complexity and scope of gene therapy products are reflected in the wide range of analytical procedures and their methods that are used to assess product quality. Approved gene therapy products must comply with applicable sections of 21 CFR 211 and 610 to ensure their identity, dose, potency, purity, and safety. Specific guidance for the identification, development, and validation of analytical methodologies to support cell and virus bank characterization, final-product release, and stability studies is currently provided in FDA guidelines for gene therapy manufacturing and testing (see *Appendix*); in *Validation of Compendial Procedures* ⟨1225⟩; and in the ICH guidelines Q2(R1) and Q6B. Most product-specific analytical methods for gene therapy products have not been standardized. Even well-defined tests such as those described under *Sterility Tests* ⟨71⟩ may not be directly applicable to certain gene therapy products. For some gene therapy products, large quantities of clinical material may not be available during early clinical development. Some required tests (e.g., sterility) may require modification. Consultation with regulatory authorities is advised.

Table 5 provides an overview of product-specific testing parameters for the biological component and general methods or approaches used to satisfy the testing requirements for nonviral, viral, and gene-modified cellular gene therapy products. The analysis of gene therapy products relies heavily on biological assays, but it also uses methodologies developed for biotechnology-derived products. The intent of this section is to outline the types of methods and their specific applications with regard to product characterization, stability, and release testing. Process validation may alleviate the need for certain specific lot-release tests. Development of appropriate reference materials and standards for viral, nonviral, and gene-modified cellular gene therapy products should be a part of product development. Reference materials should be fully characterized in order to provide continuity between standards over time. In the case of gene-modified cellular gene therapy products, the reference material may be a surrogate tissue or simulated product. Reference materials are briefly addressed in the FDA *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*.

Gene-modified cellular gene therapy products may require a rapid-release approach if they have a limited shelf life (see *Cellular and Tissue-Based Products* ⟨1046⟩). The rapid-release approach is not usually applied to viral and nonviral gene therapy products because these products are sufficiently stable for completion of testing before release. Some formulated nonviral gene therapy products also have limited shelf lives. In such cases, the individual components are tested before release and the formulated complex is not tested. The formation and stability of the formulated nonviral gene therapy complex is established via validation studies during product development.

As specified in CFR, product samples must be retained after product-release testing is completed. Retain additional samples if rapid-release strategies are employed so that product quality can be reassessed by alternative or traditional test methodologies if necessary.

Table 5. Analytical Tests for Cell and Gene Therapy Biological Products

Test	Gene-Modified Cellular Gene Therapy Product	Gene Therapy Products: Viral	Gene Therapy Products: Nonviral
Identity of Biological Substance	• Surface marker determination • Species • Morphology • Bioassay • Biochemical Marker	—Restriction enzyme map —PCR —Immunoassay for expressed gene —Sequencing	—Restriction enzyme map —PCR —Immunoassay for expressed gene —Sequencing
Dose	• Viable cell number • Enumeration of specific cell population • Total DNA • Total protein	—Particle number —Transducing units (DNA hybridization assay) —Total protein —HPLC assay using authenticated reference standard	—Plasmid–DNA weight —Formulated-complex weight HPLC or capillary electrophoresis assay using authenticated reference standard
Potency	• Viable cell number (cells intended for structural repair) • Bioassays —Colony-formation assay —Function of expressed gene — Induction of secondary effect (e.g., human leukocyte antigen (HLA) induction, secretion of cytokines, and up-regulation of surface marker)	—Function of expressed gene (induction of secondary effect and other bioassays)	—Function of expressed gene (induction of secondary effect and other bioassays)
Purity	• Percentage of viable cells • Percentage of transduced cells • Percentage of cells with specific surface marker • Process contaminants (e.g., serum)	—Residual host-cell DNA —Process contaminants (e.g., serum and cesium chloride) —Residual helper virus —Optical density ratio —Residual host-cell proteins —Viral protein profile (HPLC assay for defective or immature particles) —Residual RNA	—Percentage of specific physical form (e.g., percentage supercoiled) —Residual host-cell DNA —Residual RNA —Residual host-cell proteins —Residual solvents —Optical density ratio —Process contaminants (e.g., cesium chloride)
Safety	• Mycoplasma • Sterility • Pyrogen and endotoxins • Adventitious viruses • Residual virus • Replication-competent vector	—General safety —Sterility —Pyrogen and endotoxins —Adventitious viruses —RCV	—Mycoplasma —Sterility —Pyrogen and endotoxins

Sampling Issues

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See *Stability Protocol Development* under *Stability* (below) for additional considerations. Samples from each lot should be retained in case of a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling scheme is highlighted in safety testing for adventitious agents or in assessment of RCV for gene-modified cell or viral gene therapy products. In such cases, process validation assists in determining the appropriate statistically based sampling design.

Safety

Safety testing for gene therapy products focuses on three issues: (1) detecting contamination from adventitious sources during product processing, (2) preventing the use of packaging cell lines and plasmids that potentially permit genetic recombination between vectors and the packaging cell lines or plasmids—or among the vectors themselves, and (3) testing the final product to ensure a safe level of undesired genetic and/or structural variants or other viruses used in processing.

General Chapters

The primary means of assessing safety are the performance of biological assays to measure adventitious agents directly. Molecular biology-based assays that measure adventitious agent DNA or RNA or detect undesired genetic variants are also used. Although live genetically engineered vectors officially fall outside its scope, the detailed information available in the ICH Q5A guideline, presented in chapter ⟨1050⟩, should be consulted because the principles apply.

VIRAL GENE THERAPY PRODUCTS

One of the primary safety concerns associated with viral vectors used for gene therapy is the occurrence of undesired genetic variants. Among them the most critical type, and probably the best studied, is RCV. RCV is more clearly defined for replication-incompetent viral vectors, but for conditionally replication-competent viruses it refers to undesired genetic variants that have lost selectivity toward the target cells and thus might raise safety concerns. Regardless of the virus, these concerns are based on the potential lack of predictability for the pathogenicity of a contaminating virus for a specific route of administration, particularly if it is not the normal route of infection or if humans are not a natural host for the virus. The pathogenesis of a wild-type adenovirus infection is known but may not be predictive for the routes of administration employed with recombinant adenoviral vectors. For replication-incompetent adenoviral vectors, a limit of one RCA per 3×10^{10} viral particles is currently considered acceptable (see the FDA *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*).

Typically, RCA levels are determined by a cell-based assay that allows amplification of the RCA while preventing replication of the product. The cell line most often used for amplification and detection of RCA is the A549 cell line. However, some recombinant adenoviral vectors express therapeutic genes that interfere with analysis on A549 cells. In such cases, a bioassay-based two cell lines is used. The first cell line is chosen on the basis of resistance to the effects of expression of the transgene and with subsequent passage of cell lysate or supernatant onto A549 cells for amplification and detection of the RCA. RCA is most often detected by visual observation of the cytopathic effect, but it can also be detected in the A549 cell culture by immuno- or PCR-based methods.

Quantitation of the RCA level is based on the quantity of sample tested and the detection limit of the assay. Typically, RCA bioassays are validated as being able to detect 1 plaque-forming unit or infectious unit of RCA in the test sample over a wide range of test-sample sizes. Test-sample sizes can range, but they are typically based on the FDA RCA acceptance limit. To verify detection limits, include spike controls as part of the test, even with validated assays. For recombinant adenoviruses produced using HEK293 cells, RCA detection by PCR on the final products or the progeny virus amplified in HEK293 cells can be confounded by detection of residual HEK293 host-cell DNA (detection of the E1 region). PCR assays, however, can be designed to specifically quantitate host cell DNA contamination and can be made specific to particular forms of slow-growing RCA. Quantitative PCR assays can be used in conjunction with a cell-based method for precise quantitation of RCA levels. When a tested sample is found to be positive, the identity of the RCA is usually confirmed by conducting PCR analysis. This rules out the possibility that contamination of the assay by exogenous wild-type adenovirus or other adventitious agents is responsible for the positive result.

For conditionally replication-competent adenoviruses or other replication-competent viral vectors, testing for RCV or undesired genetic variants is usually more complicated and vector specific. Usually one or two nonpermissive cell lines that are not target cells are infected with the replication-competent virus in attempts to produce progeny virus. In order to generate a sufficient quantity of progeny population for analysis, analysts subject the infection to multiple passages and extended culture time. Two normal fibroblast cell lines that are easy to culture, WI-38 and MRC-5, have been used as the model nonpermissive cell lines for detecting RCA in replication-competent adenovirus products. Even after multiple passages on the nonpermissive cell lines, it may be necessary to amplify the progeny (which tend to appear only in minute quantities) in permissive or packaging cell lines to a sufficient quantity for subsequent testing. The resulting progeny should be tested for changes in biological selectivity and genetic composition. Usually the genetic characterization of the progeny population includes restriction enzyme mapping followed by Southern blotting, PCR, or nucleotide sequencing. After the genetic elements unique to RCV or undesired genetic variants are identified, quantitative PCR assays can be designed to monitor the level of RCV after amplification in nonpermissive cell lines or sometimes, if the sensitivity is adequate, directly in the final product without biological amplification. Using a spike control in the biological assay for detecting RCV is encouraged but may not be applicable to all cases. Currently there is no specified acceptable limit of RCA for conditionally replication-competent adenovirus, although clinical safety has been reported for an oncology application with several thousands of RCA per dose.

For retroviral vectors, testing for RCR is required for cell banks, viral vector production lots, and any resulting ex vivo product lots (see FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*). Standard assays have been designed to detect replication-competent MLV. The pathogenesis and potential long-term toxicity of low-level amphotropic MLV in human beings is not known. Methods commonly used to detect RCR include an amplification of virus titer by application of product to a replication-permissive cell line such as Mus dunni. Because infection is limited by the ability of a virus to reach the cells by means of Brownian motion, procedures (e.g., centrifugation and filtration) that physically bring the virus into contact with the cells can be used to enhance detection. However, high-titer recombinant vector can interfere with the detection of low-level RCR, and this interference may be enhanced by such methods. Infected cells are passaged several times to allow viral replication. Culture medium is harvested at the end of the culture period, and RCR is detected by using an indicator cell line. If the product is an amphotropic MLV, RCR can be detected by using a feline cell-based PG4 S+L– assay, a

General Chapters

mink cell-based MiCl S+L– assay, or a marker rescue assay. In S+L– assays, the RCR expresses proteins that lead to transformation and subsequent plaque formation on the monolayer. In a marker rescue assay, RCR infects a cell line that expresses a retroviral vector encoding a marker gene such as β -galactosidase, drug resistance, or a fluorescent protein. The vector is packaged by the proteins supplied to it in trans by the RCR. The potentially vector-laden supernatant is transferred to naive target cells that are then screened for expression of the marker vector.

Testing for RCR is performed by co-cultivation of the cell line or amplification of vector supernatant with an RCR replication-permissive cell line, typically *M. dunni*, for several passages. Culture medium is harvested at the end of this co-cultivation process and applied to an appropriate indicator cell line as described above. Note that artifacts may be generated during the co-cultivation assay by expression of an endogenous virus in the permissive cell line or by fusion if the vector-producing cell line is cultured directly with a marker rescue cell line. In addition, co-cultivation may not be possible for ex vivo cell products that have specific culture requirements or limited culture life spans.

Methodologies for testing the presence of RCR in crude, purified bulk or final vector products are not specified. FDA has deposited a reference standard of an amphotropic hybrid MLV with the American Type Culture Collection (ATCC). This viral stock has been assigned a label titer and should be used in assay validation. Method validation should demonstrate the ability to reproducibly detect a single RCR particle in individual product types because the product and its related impurities can interfere with the detection of RCR. Currently, there are no acceptable limits for RCR contamination in products. Any product lot found to contain RCR cannot be used for human use. Reference standards for assessing RCV in other viral vectors including ecotropic, xenotropic, or pseudotyped MLV, adenovirus, and lentivirus have not been developed. The adenovirus reference material, which consists of wild type human adenovirus type 5, has been used as a spike control and during validation of RCA assays, but this practice may not be applicable to all RCA assays. Amplification and detection of replication-competent HIV, especially its pseudotyped variants, may warrant special containment and handling procedures.

Additional safety testing usually focuses on methods similar to those described in *Biological Reactivity Tests, In Vivo* ⟨88⟩, *Safety Tests—Biologicals*, and chapter ⟨71⟩. For viral gene therapy vectors produced using a human cell line, performance of the in vitro adventitious agent bioassays using three cell lines is recommended. For adenoviral vectors, specific tests for adeno-associated virus are also recommended. For adeno-associated virus, specific tests for adenovirus and herpes virus are recommended. Material for testing should be derived from the stage of manufacture that provides the greatest chance of detection, which could be pre-bulk (e.g., late-stage fermentation), the bulk, or the final product.

NONVIRAL GENE THERAPY PRODUCTS

Safety testing usually focuses on methods similar to those described in chapters ⟨88⟩ and ⟨71⟩. Safety testing should be performed on nonviral formulated material. If the shelf life of the formulated nonviral product is very short, then the components should be tested individually.

Safety testing for undesired genetic variants that might emerge during the manufacturing process in nonviral gene therapy products is similar to that for RCV testing for viral vectors but with more vector-specific considerations. Typically, molecular biology-based methods are applied to the final product to test for variants. When genetic stability is established by process validation, the assays for monitoring the levels of undesired genetic variants may be limited to restriction enzyme mapping followed by further confirmation of critical genetic elements (such as transgenes or regulatory elements) by PCR or Southern blotting.

Dose-Defining Assays

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision. An assay that measures therapeutic activity of the product is referred to as a potency assay, and it is designed to measure product function. The design of the assay depends on the type of product. In the case of chemical and protein drugs, the assays measuring the amount of active ingredient (dose) are referred to as strength assays. Product dose can be defined as the concentration or amount of the drug product administered to the patient, and it is typically measured as product mass.

Particle concentration is a commonly used measure for viral vector product dose. Particle concentration may be measured by physical, biophysical, or in vitro cell-based assays. For example, quantitation of purified adenoviral particles may be determined by using the optical density of a solution of virus in 0.1% (w/v) sodium dodecyl sulfate (SDS) solution at 260 nm, because a relationship between absorption and particle concentration has been published for adenovirus. The particle number concentration is equivalent to the product of the absorbance at 260 nm in a 1-cm cell, the dilution factor, and 1.1×10^{12} particles. A method that has become standard in determining particle concentration is integration of viral peak area of 260 nm and/or 280 nm absorbance against an authenticated reference standard in an anion-exchange resin-based high-performance liquid chromatographic (HPLC) assay. Compared to the optical density method, the HPLC method has the advantage of eliminating the interference of free DNA and/or capsid proteins on quantitation of viral particles. An adenoviral reference material (ARM) from ATCC has an HPLC-determined particle concentration established from a large-scale collaboration that involved many laboratories. Whenever possible, the ARM should be used to calibrate the internal HPLC method and reference material.

Virus concentration can also be assessed by the measurement of selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus must be lysed, and the structural proteins must be sep-

arated by using an appropriate, high-recovery chromatographic procedure (e.g., reversed-phase HPLC). The chromatographic separation and the identity and the purity of the selected structural protein must be verified during assay validation by methods such as SDS polyacrylamide gel electrophoresis (SDS-PAGE), peptide sequencing, and mass spectroscopy. The selected structural proteins can be quantified, for example, by integrating chromatographic peaks at 214 nm and comparing the area to that of an authenticated reference standard. The virus concentration can then be calculated based on the molecular mass, the copy number, and the measured mass of the protein. Very importantly, the virus concentration can be estimated simultaneously for multiple structural proteins, which allows the use of this assay in relatively impure virus preparations. This method has been applied to adenovirus and should be applicable to other viral vector types.

Biophysical methods of determining particle number include direct quantitation of vector nucleic acid by radiolabeled-probe hybridization and indirect quantitation by amplification of template nucleic acid (e.g., PCR and RT-PCR) or by signal amplification (e.g., branched-chain DNA using multiple-probe hybridization).

In cases where biophysical methods are not available, bioassays that measure gene-vector titer have been used. These involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some measure of the product uptake. Methods for quantitation or estimation of the number of infection, transfection, or transduction events include plaque-forming unit assays, tissue culture infectious dose assays based on cytopathic effect of 50% ($TCID_{50}$) or immunofluorescent detection of an expressed vector protein, or a quantitative DNA-hybridization assay. Examples follow: For replication-competent adenoviral gene therapy products, the ARM available from ATCC has a defined range of $TCID_{50}$ titer determined via a collaborative effort. Whenever applicable, it should be used in validation of an internal reference standard or assay control of infectious titer assays. However, because of the likelihood of genetic differences between the ARM, which is wild-type human adenovirus type 5, and the replication-competent adenoviral gene therapy product, it may not be reasonable to normalize the titer of the vector of interest to that of the ARM.

For retroviral or lentiviral gene therapy products or AAVs that carry a selectable marker (e.g., that for neomycin resistance) or a reporter gene (e.g., β-galactosidase) in addition to the therapeutic gene, the infectious titer is commonly determined by measuring the number of transduced or infected cells expressing these nontherapeutic proteins. Vector titer is typically reported as the number of colony-forming units (cfu) per mL for cells transduced with viral vectors that contain drug-resistance markers and are selected for growth in drug-containing medium. Titer based on β-galactosidase can be expressed in terms of blue cfu per mL after staining and counting the cells that convert the β-galactosidase substrate X-Gal into a blue chromophore. For vectors without a marker gene, quantitation of transduction has been measured precisely by using quantitative PCR or has been estimated by hybridization methods.

Most nonviral gene therapy products contain plasmid DNA, and their usual measure of dose is the DNA mass. The DNA mass may be determined in the formulated state, and, if recombinant protein is included in the formulation, the total combined mass of all formulation components based on a specific ratio can be used. DNA concentrations greater than 500 ng/mL are most simply determined by using optical density measurement at 260 nm. This method is not generally applicable to lipid-formulated DNA. Because RNA and proteins also have significant absorbance at 260 nm, other analyses must be performed to demonstrate minimal contamination with RNA, protein, or residual host-cell chromosomal DNA. Dyes that specifically bind to double-stranded DNA allow accurate measurement of DNA concentrations of less than 500 ng/mL when calculated against an authenticated DNA standard curve. PicoGreen is one such fluorescent dye, and it is minimally affected by single-stranded DNA, RNA, proteins, salts, and detergents. The fluorescent dye Hoechst 33258 also binds to both double-stranded and single-stranded DNA and it can be used to determine DNA concentrations as low as 0.3 ng per mL. The Hoechst 33258 does not bind to protein or RNA, and it can accurately determine the DNA concentrations in crude samples.

Methods such as capillary electrophoresis and HPLC employing an authenticated reference material can also be used to determine the strength of nonviral products.

Potency

Potency is defined as the therapeutic activity of the drug product. Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*). Potency can be assessed by in vitro or in vivo bioassays. It is not uncommon for these assays to have coefficients of variation between 30% and 50%, although stringent assay design with good statistical consideration could help reduce assay variation. These assays require a well-defined, representative reference material that can be used as a positive control for the assay and/or in calculation of the relative potency of the test article. The general consideration for bioassays in current *USP* chapters on design and development of biological assays should be applied to the potency assay design for gene therapy products. The positive control qualifies the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. High-precision assays are more effective tools in monitoring product quality. Information about potency assay variability should be incorporated into the stability study design and the proposed statistical approach to assignment of expiration date (see *Stability*).

Bioassays employed to measure the potency of viral and nonviral gene therapy products generally involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some functional measure of the expressed gene of interest. Functional assays for the therapeutic gene (e.g., those measuring enzyme activity and cell growth stimulation or inhibition) should generally be used instead of analytical methods such as enzyme-linked immunosorbant assay (ELISA). When the biological function of the expressed transgene exhibits a broad range of activities or only generates semiquantitative results, the ELISA or other immunological or biochemical readouts can be used as a surrogate potency assay with a tight specification range if

extensive characterization data is available to demonstrate that all expressed protein is biologically active. For example, in the case of a gene therapy product expressing a cytokine, cytokine expression is usually quantified by ELISA first, and the result is used to adjust the sample dilution for the functional assay. The potency of such vectors may be better controlled by the ELISA quantitation results, but the biological activity of expressed cytokine could be used to verify that the measured mass is biologically active without the requirement to meet a narrow specification range for the biological activity itself.

HPLC or flow cytometry, which provide information about the level of expression but only infer function, have also been used in a context like that described for immunoassays. In addition, for viral vectors, infectious titer measurements by themselves are generally not considered an adequate measure of product potency. For example, the TCID50 titer or plaque-forming-unit assays for adenoviral vectors on HEK293 cells can indicate that the infectivity of adenovirus is preserved but do not confirm that the adenoviral product has maintained full biological function(s), especially transgene biological activity. The design and ultimate suitability of an assay system for determining product potency depends on the relationship between the intended human target cell in vivo and the following: (1) the transduction or transfection efficiency of the cell line used in vitro; (2) the protein expression levels; and, (3) the duration of expression required for the therapeutic effect.

In vivo tests can also be used to measure vector-product potency. Readouts can be based on a response per animal (e.g., blood levels of therapeutic protein 24 hours after treatment) or a group response rate (e.g., percentage of animals that elicited an immune response or survived virus challenge). The availability of an appropriate in vivo test system depends on the vector-host range (for viral vectors), the pharmacokinetics and biodistribution of the vector and the resulting gene product relative to its human counterpart, and the time frame required to observe the therapeutic effect or surrogate. Issues of cost, facilities, validation, and ethics determine the practicality of an in vivo potency test.

Purity

Analytical methods that separate, isolate, and specifically quantify the intended active product components determine product purity. Impurities are either product- or process-related components that can be carried through to the final product. The manufacturing and purification process should be optimized to consistently remove impurities while retaining product activity. The requirement to test for a particular impurity for product lot release depends on the following: (1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity through process validation, and (2) the potential toxicity associated with the impurity.

Examples of process-related impurities associated with gene therapy products include residual production-medium components (e.g., FBS, antibiotics, cytokines, and *E. coli* chromosomal DNA in a plasmid product), ancillary products used in downstream processing (e.g., nucleases such as DNase I), and residual moisture for lyophilized vector products. Impurities may be bioactive (e.g., cytokines and hormones) or immunogenic (e.g., product aggregates, degradation products, plasmid-selection markers, and nonhuman-derived proteins), or they may have other deleterious effects (e.g., they may compete with the product) if administered at a dose equal to that of the product. Product-related impurities are specific to each product type. Examples include nicked plasmid forms in nonviral products and defective or immature virus particles in retroviral or adenoviral vector products. Analytical methodologies to assess purity require quantitation or physical separation of intended product from its impurities. Common sense should drive the need to quantify specific impurities. Suitable validation of the manufacturing process may limit the need for specific lot-release testing for impurities. Manufacturers may place an emphasis on demonstrating the consistency of the product-impurity profile.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

VIRAL GENE THERAPY PRODUCTS

Product-related impurities for viral vectors include aggregates and defective and immature particles that may be produced during the manufacture or purification of the recombinant vector. Aggregates of vector may form if the product is highly concentrated, stored under certain conditions (e.g., under a certain pH or temperature), or reconstituted after lyophilization. Assays to detect aggregates include particle size analysis by laser light-scattering and the use of nonreducing, nondenaturing PAGE, followed by staining of the gel or transfer and detection of viral proteins by Western blot analysis. Sedimentation rate analysis also allows separation of aggregates from monomers based on size. Optical density analyses of light scattering are also used to assess vector aggregation.

Defective particles are viral particles that do not contain the appropriate recombinant genome—that is, they contain some other nucleic acid or contain no genome at all, or the vector has some missing, defective, or otherwise altered structural component that impairs its ability to transduce a cell. For viral vector systems that have capsomeric symmetry that requires the appropriate nucleic acid incorporation for configuration, empty particles may be readily distinguished from those carrying genomes. For enveloped viruses, empty particles may not be as readily separated from those with encapsidated nucleic acid.

For some viral vector products, active viral particles can be separated from defective particles by using analytical HPLC. Anion-exchange resins have been used to separate active adenovirus from defective virus particles. However, this method might not be useful for an adenoviral vector purified by anion-exchange chromatography unless the resin for the assay is different from that used during manufacture. Depending on the nature of the viral vector and its nonactive or defective forms,

other methods of separation, such as equilibrium centrifugation in a cesium chloride density gradient, may need to precede the quantitation of the active particle. Ideally, the method of separation will allow quantitation.

Defective particles that carry a non-cell-derived oncogene or other undesirable genes may pose a special concern. For example, in murine-based retroviral packaging cell lines, small viral elements called VL30 sequences can be packaged in about one-third of all particles. Assays may be needed to quantify specific defective particles if they are known to be present in quantities sufficient to pose a safety concern.

Virus quality and the comparability of preparations can also be assessed by measuring selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus is lysed, and the structural proteins are separated by using reverse-phase HPLC or some other high-recovery chromatographic procedure. The chromatographic separation should be validated, and the identity of the selected structural proteins should be verified by methods such as SDS-PAGE, peptide sequencing, or mass spectroscopy. Fingerprinting of the batch can be conducted based on quantification of the selected structural proteins and comparison to a reference standard. When the method incorporates mass spectroscopy, impurities such as structural variants can also be identified. For adenovirus preparations, some precursor and most mature virion proteins can be detected and distinguished, thus allowing monitoring of the product and of the immature virion forms.

Host cell-derived proteins may be considered impurities for some viral vector products and may be separated and quantified by PAGE or HPLC or detected by amino acid analysis, Western blot, or immunoassay-based methods. However, for enveloped viruses such as retroviruses, host cell-derived membrane proteins are an integral part of the product. In those vector systems, it may be difficult to determine the presence of contaminating exogenous host-derived proteins.

Presence of specific process-related impurities depends on the manufacture and purification process of each vector or product type. However, most products need to be tested for residual endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩). Acceptable limits of endotoxins have been determined and can be directly applied to viral vector products. Although genomic DNA derived from continuous cell substrates used to manufacture biological product historically has been considered potentially tumorigenic, recent studies suggest that the risks are very low. However, every attempt should be made during process development to reduce levels of contaminating DNA. The need to test for residual DNA as part of product lot release should be evaluated on a case-by-case basis and may depend on the size distribution of the DNA, its association with the product or its formulation components, and the product's route of administration. Quantitative PCR assays can analyze the amount of residual host-cell DNA by using primers designed to amplify evolutionarily conserved and abundant target sequences such as 18S for HEK293 cells.

Quantitation of residual serum components such as bovine serum albumin (BSA) can be achieved by using ELISA and a BSA reference standard. Researchers may need to develop specific functional or immunological methods for other ancillary products, including other culture media or purification process components such as cytokines or enzymes (e.g., deoxyribonuclease 1 or benzon nuclease).

NONVIRAL GENE THERAPY PRODUCTS

A plasmid used as a drug substance is considered a well-characterized biologic, and key impurities from the manufacturing process are well known. Testing is usually performed on each individual component: the plasmid DNA, lipid or lipoplex reagents, and protein components if any are present in the formulation. Plasmid DNA is characterized for a variety of impurities, including residual host-cell DNA, residual RNA, and residual protein. Residual protein testing is frequently included in lot-release testing. Optical density ratios (usually the measurement at 260 nm to that at 280 nm) are frequently used in purity specifications for plasmid DNA.

In addition, the plasmid DNA should also be characterized with regard to its conformation in solution. Plasmid DNA exists as monomeric supercoiled, relaxed monomer, and linear forms. Because all forms can be generated during large-scale fermentation, and data about their relative in vivo potency is scarce, the relative quantity of each form is monitored to verify batch-to-batch consistency in the relative amounts of each conformation. Agarose gel electrophoresis can resolve each of these forms but is not highly quantitative for each individual species. Analytical anion-exchange HPLC serves as a quantitative assay for monomeric supercoil and other forms, including concatamers. Other analytical methods that have been valuable for characterization of plasmid constructs during process development and validation such as capillary gel electrophoresis, linear-flow dichroism, and atomic-force microscopy are also viable methods to assess the purity of a given plasmid preparation. The most appropriate method for lot release depends on how each plasmid conformation affects product potency. Specific details for each of these methods are outlined in *Nucleic Acid-Based Techniques—General* ⟨1125⟩.

Tests should be conducted for process-related impurities such as residual organic solvents (phenol, alcohol), salts, and certain antibiotics such as kanamycin used during the fermentation process. Lipid and lipoplex formulation components must also be tested for their chemical purity. Testing for specific chemical impurities is commonly performed by using gas chromatography–mass spectroscopy (GC–MS), HPLC, or thin-layer chromatography (TLC) methods. If protein is part of the formulated complex, then the protein must also be tested for purity. HPLC is capable of detecting trace amounts of residual antibiotics and can therefore be used during process validation or lot-release testing to confirm that they have been effectively removed. The specifics of these methods are outlined in *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩ or in *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

General Chapters

Bacterial protein, DNA, RNA, and endotoxins are the major types of host-derived process contaminants. Standard protein assays (e.g., Lowry, Bradford, or Coomassie), PAGE followed by silver staining or Western blot analysis, or ELISA can be used to detect residual host protein in the nanogram range. Host chromosomal DNA can be detected by slot blot hybridization (detection in picogram range) or by real-time PCR (detection sensitivity < 1 pg) using highly conserved target sequences (e.g., 18S for *E. coli*). PCR assays for this purpose must use recombinant polymerases that are highly purified to minimize residual bacterial DNA for which the presence can create background signals. PAGE or agarose gel electrophoresis followed by fluorescent dye staining can be used to detect residual RNA. Quantitation may not be required because of the labile nature of RNA and the low-level toxicity associated with it. The *Limulus* amoebocyte lysate (LAL) test is the most sensitive and widely used method for endotoxins determination. Colorimetric assays offer sensitivities of 0.005 EU/mL. Details of the methods described here are outlined in 〈1057〉, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* 〈1056〉, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids* 〈1130〉, 〈1125〉, and 〈85〉.

LYOPHILIZED VIRAL AND NONVIRAL VECTOR PRODUCTS

Residual moisture can affect the stability of a lyophilized vector product. FDA's *Guideline for the Determination of Residual Moisture in Dry Biological Products* recommends a 1% residual moisture level, although data indicating no adverse effects on product stability at higher levels is considered acceptable. Residual moisture levels can be determined by using a standard method (see *Water Determination* 〈921〉) that is compatible with the formulated product.

Identity

Lot-release testing for gene therapy products must include an identity test. This test clearly identifies the product and confirms that inadvertent substitution with another product has not occurred. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous gene-modified cell therapy product at a facility where multiple patient products are manufactured than for a viral vector product produced at a site that manufactures a single vector product.

VIRAL GENE THERAPY PRODUCTS

For characterization purposes, restriction enzyme mapping and sequencing of the transcription unit DNA are the most commonly used approaches. PCR-based methods, restriction enzyme mapping, and transgene-expression-based immunoassays are commonly used to confirm the identity during lot-release testing.

NONVIRAL GENE THERAPY PRODUCTS

Restriction enzyme mapping is the most common identity method for plasmid-based products. The number of enzymes used to create the vector fingerprint will vary according to the complexity of the DNA and the degree of similarity between multiple products. If lipids, lipoplex agents, or proteins are used to formulate the DNA, then their identity must also be tested and confirmed. Lipids and lipoplex components may be identified by procedures used for traditional pharmaceuticals, such as GC–MS and TLC. Protein components of the formulation may be identified by peptide mapping or other means outlined under 〈1057〉.

STABILITY

The shelf lives of gene therapy products vary widely depending on the nature of the product, its intended clinical use, its specific attributes, and the recommended storage, packaging, and shipping conditions. Therefore, it is difficult to draft uniform guidelines regarding stability-study duration and testing frequency for all products. In all cases, the study should be designed on the basis of scientifically sound principles and approaches and a comprehensive understanding of the final therapeutic product and its intended use. The product's stability during in-process hold steps, cell and virus banks, critical raw materials, and reference standards also must be assessed. A well-designed and executed stability program will provide a high degree of assurance that the product is stable within the specified shelf life.

For viral and nonviral vector gene therapy products and gene-modified cellular gene therapy products that are not patient specific, the selection of batches to support license application and final-product labeling should be carried out in accordance with the principles of stability testing, such as those described in ICH guideline Q5C and chapter 〈1049〉. Stability data should also be collected for bulk material and at other in-process points if material is stored before final processing and filling. Issues related to the stability of cell-based products are addressed in chapter 〈1046〉.

Nonviral DNA plasmid vectors are often formulated with specific mixtures of lipids, proteins, or lipoconjugates to form liposomes or encapsulated complexes. Depending on the formulation, a shelf life of hours to years can be attained. Where a product has a short shelf life, the final formulation may require preparation at the clinic just before administration. Instability is frequently observed as aggregation and precipitation. Formation and stability of the formulated complex should be characterized

and established by validation studies during product development. Stability data should also be collected for major components of the formulated complex, such as the lipids, the liposomes, and the DNA itself.

Stability-Protocol Development

Stability studies verify that the storage conditions maintain the purity and potency of the product for a defined period so that product administered to the patient is still capable of meeting the stability specifications. These specifications may differ from the manufacturing release specifications, but they must be verified with clinical data. Formal stability studies to support licensure as well as plans for gathering early-phase product stability information should be detailed in a written plan that describes how data will be collected and analyzed to support the product's expiration period. Protocols should follow the format recommended in existing guidelines and should include the scope, storage conditions, number of lots that will be tested, test schedule, assays that will be used, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the problems the product may encounter during manufacturing, shipping, and processing at the clinical site (see *Accelerated and Most Appropriate Challenge Conditions*, below). The study design should also incorporate the latest knowledge in the biological sciences and should address existing regulatory requirements. For instance, if the product's final formulation is performed at the clinical site, stability studies on this final formulation should be performed to establish the time and conditions under which the product can be held.

Stability assessment should include an evaluation of product functionality (potency). The potency assay often has a high degree of inherent variability. Measuring and calculating the decay of product activity by employing the standard statistical methodologies may require multiple, frequent sampling intervals over an extended period of time and may require analysis of more than three production lots to compensate for assay variability. Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Initial studies are also useful for determining which assays are stability indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of gene therapy products, the development of assays that would address these unique characteristics is encouraged.

Accelerated and Most Appropriate Challenge Conditions

The stability-indicating profile of a gene therapy product varies over time under the influence of a wide variety of environmental conditions, including temperature, extremes in physiological storage conditions, and light. Multifactorial degradation pathways must be considered when researchers investigate the effects of these parameters on the stability of the product. Studies should include conditions that are outside the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation due to shipping to hot or cold climates, hypobaric conditions experienced in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite. A short exposure to an environmental condition well outside of an established limit and a long exposure to an environmental condition just outside of an established acceptable range may be equally detrimental to the overall stability profile. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Give special attention to products stored in fluids that contain light-sensitive or reactive components that may give rise to cytotoxic byproducts.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to determine how the product degrades and which assays are stability indicating. These studies can be the same as some of those mentioned in the preceding paragraph. Other studies include placing a product at 37°, or at 18° when its normal storage temperature is 25 ± 2°, or placing a lyophilized product in a high-humidity environment. Such studies should be performed before formal stability studies begin so that the latter can incorporate the validated stability-indicating assays.

STORAGE AND SHIPPING

Appropriate conditions are chosen to preserve the purity and potency of the product so that its specification and those of its ingredients are maintained throughout storage, shipping, and handling at the clinic. Initial studies must be conducted before patient administration to determine acceptable storage, shipping, and handling conditions. The initial storage and shipping conditions need not be those envisioned for the commercial product but should ensure that the product specifications are maintained beyond the initial expiration date. For products with short shelf lives, storage and shipping conditions, even within a medical center, must be considered at the same time because shipping constitutes the bulk of storage time after manufacturing. Give special consideration to the ability of gas to permeate the shipping container, especially if the gene therapy product is stored or shipped on dry ice. Once stability-indicating methods are developed and the final storage and shipping conditions are chosen, these must be validated as discussed under *Stability*.

General Chapters

Most products with limited shelf lives are shipped by reliable overnight courier systems. In some cases, highly fragile products are hand-carried onto commercial aircraft. Special permission must be obtained by commercial carriers if scanning by airport X-ray equipment must be avoided. Cargo shipping studies should be designed during the development of packaging systems to identify stresses to which the product may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will tolerate, and protect the product against, the extreme conditions of shipping.

Most gene therapy products can be either lyophilized or formulated by means similar to those employed for many recombinant proteins or cell therapy products. These storage formulations typically have expiration periods longer than one year and no unusual shipping requirements. Nonviral gene therapy products, which may be unstable in their final formulation, can have similar expiration periods if they are stored in a multiple-vial kit with the nucleic acid in one vial and a carrier, such as lipids, in the other. The final formulation is performed at the medical center just before administration.

LABELING

Product labeling is regulated by the FDA, and compliance with existing policies is required. Because gene therapy products are regulated biologics, their labeling is subject to these rules. Biologics and devices must meet labeling requirements specific to the container and the package (21 CFR 610 and 801, respectively). Both the container label and the package label must include the expiration date. If the container is packaged, then the recommended storage conditions should be included on the outer package label. If the container is not packaged, the recommended storage conditions and all other requirements of a package label must appear on the container. Labeling must also comply with relevant national and international requirements.

If a product must be applied to the patient in a particular physical orientation or in a specifically defined area, labeling that indicates the correct orientation and/or area should be apparent even after the package is opened. Unless the product has been screened for pathogenic or microbial contaminants before release, appropriate biohazard labeling may be required. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life. Clinical procedures must be scheduled around these crucial time frames. For patient-specific products, the patient's full name, initials, or a combination of these will need to appear on the labeling, in addition to lot designation, to ensure that the product is administered to the appropriate patient.

REGULATIONS AND STANDARDS

The technologies involved in manufacturing gene therapy products have been widely documented in the literature and continue to evolve. These products can be regulated as drugs or biologics, or uncommonly as devices, depending on how they are manufactured and used. The novel approaches permitted by these technologies may make it difficult to determine which FDA centers will be involved in their regulation, and the FDA has advised manufacturers to seek clarification in the early stages of development. Currently, the Center for Biologics Evaluation and Research (CBER) regulates most human gene therapy products. CBER relies on both the Public Health Service Act and the Federal Food Drug and Cosmetic Act. Regulation is the same as that for biotechnology-derived products. The general requirements are described primarily in 21 CFR. The federal government has issued many guidance documents as *Points to Consider* or *Guidelines* (see www.fda.gov). ICH guidance documents for many of the quality-related areas are relevant in varying degrees to qualifying gene therapy products (although some products are nominally outside the scope of the guidance documents, the principles still apply; see www.ifpma.org or www.ich.org). Some of these documents are reproduced in *USP–NF* as general chapters. ICH has also held a number of meetings about gene therapy products and has a Gene Therapy Discussion Group (GTDG) that addresses current issues in gene therapy product development and research and has released several ICH Considerations that reflect harmonized principles. The National Institutes of Health (NIH) has published *Guidelines for Research Involving Recombinant DNA Molecules* that require NIH review of research, including clinical research or trials conducted or sponsored by institutions that receive NIH funding. These guidelines apply to many gene therapy products.

Biological and biochemical standards for QA of the production and analysis of gene therapy products are highly desirable. The diversity of gene therapy products, in particular viral vectors, has so far limited the development of standards that have wide applicability. A MuLV RCR preparation (VR-1450) with an assigned infectivity titer is available from ATCC for testing murine retroviral vectors for the presence of RCR. A wild-type adenovirus type 5 reference standard with assigned particle number and infectivity titer for characterization of adenoviral vectors is also available from ATCC. A working group has been set up to oversee the development of an AAV reference standard. However, several obstacles to choosing, developing, establishing, and circulating suitable standards are apparent. These include decisions about which virus serotype will be most commonly and successfully used for gene therapy, availability of GMP prepared materials, safety, long-term stability, transportation, and initiation and completion of collaborative studies to evaluate candidate standards. Thus, development of standards for other viral vectors, including lentiviral-, herpes viral-, and poxviral-vectors, remains challenging.

New methodologies, including proteomics, novel nucleic acid technologies (NATs), protein modification methods, and stem cell isolation and culture, are now available and, in many cases, are applicable to the development, characterization, and analysis of gene therapy products. In addition, the use of synthetic polymers both for the modification of existing viral vectors and for the development of chemically dynamic synthetic vectors provides advantages, e.g., improved systemic circulation, better targeting and delivery, and lower levels of immunostimulation and inflammation. The availability of defined stem cell popula-

tions and improved engrafting methods should lead to greater effectiveness of ex vivo transduced cells used in gene therapy protocols. The introduction of new methodologies will require the continual review and regulatory oversight to ensure the quality and safety of gene therapy products of the future.

GLOSSARY

Adenovirus: Virus belonging to the family Adenoviridae of DNA viruses having a nonenveloped virion with 252 capsomeres and a diameter between 70 and 90 nm; a single linear molecule of double-stranded DNA (36 to 38 kb); at least 10 structural ether-resistant and acid-stable proteins; virions are released by cell destruction.
Adenovirus-Associated Virus (AAV): Human parvovirus contains a single-stranded DNA genome and depends on helper viruses (adenovirus, herpes virus, or vaccinia-virus) for replication. Without co-infection, the wild-type virions integrate at a specific site on chromosome 19 and remain latent.
Adventitious Agent: A foreign agent that is introduced accidentally or inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).
Amphotropic Virus: A virus that infects and replicates in cells from multiple species.
Ancillary Materials: Components used during manufacturing that are not intended to be present in the final product. Examples: growth factors, monoclonal antibodies, cell-separation devices, and media components.
Autologous: From one's own body.
Base Pair: Two nucleotide bases on different strands of the nucleic acid molecule that bond together.
Bioassay: Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*.)
Biological Product: Any virus, therapeutic serum, toxin, antitoxin, or *analogous product* applicable to the prevention, treatment, or cure of diseases or injuries in humans. (In this US FDA-derived definition, the term analogous product has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)
Biotechnology: Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.
Cell Lines: Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or may be immortalized (modified to be able to replicate indefinitely).
Cell Therapy: Therapy that uses whole cells to treat a disease, condition, or injury. Distinct from tissue and organ transplantation.
CFTR: Cystic fibrosis transmembrane conductance regulator.
cGMP: Current good manufacturing practices. FDA outlined cGMPs in the 21 CFR, and in the *Federal Register*, and its *Points to Consider*.
Complementary DNA (cDNA): DNA synthesized from an mRNA rather than a DNA template. It is used for cloning or as a DNA probe for locating specific genes.
Cytokine: Any factor that acts on cells; usually a protein that promotes growth.
Cytoplasm: Cellular material that is within the cell membrane and surrounds the nucleus.
Cytotoxic: Able to cause cell death.
Differentiation: A process of biochemical and structural changes by which cells become specialized in form and function.
Ecotropic Virus: A virus that infects and replicates in cells from only the original host species.
Electroporation: Method for enabling transfer of material into cells that involves use of a brief electrical field to create temporary pores in the cell membrane.
ELISA: Enzyme-linked immunosorbent assay. An immunoassay that utilizes an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.
Enveloped Viruses: Viruses containing a lipoprotein bilayer surrounding the capsid and acquired by budding through the cell membrane of the host cells.
Episomal: Pertaining to any accessory extra-chromosomal genetic material.
Ex Vivo: Procedure performed outside of the living organism.
Fluorescence-Activated Cell Sorter (FACS)——: A machine that sorts cells based on fluorescent markers attached to them.
Formulated: Prepared in accordance with a prescribed method or conditions.
Fusion: Joining of the membrane of two cells, creating a daughter that contains some of the same properties from each parent cell. It is used in making hybridoma cells in which antibody-producing cells are fused to mouse myeloma cells.
Gene Construct: Expression vector that contains the coding sequence of the protein and the necessary elements for its expression.
Gene Therapy: Therapy that uses nucleic acid that is subsequently expressed as RNA or protein to treat a disease or condition. The US FDA defines gene therapy products as products containing genetic material administered to modify or manipulate the expression of genetic material to alter the biological properties of living cells.
Genome: Total hereditary material of a cell.
Germ Cell: Reproductive cell (sperm or egg), gamete, or sex cell.

Graft-Versus-Host Disease (GVHD): Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.
Growth Factors: Factors responsible for regulatory cell proliferation, function, and differentiation.
Helper Virus: Aids the development of a defective virus by supplying or restoring the activity of a viral gene or by enabling the defective virus to form a functional envelope.
Hematopoietic: Pertaining to or affecting the formation of blood cells.
Hepatocyte: The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells generally do not divide, but when injured they can divide and regenerate until the injured cells are replaced.
Herpes Simplex Virus (HSV): A DNA virus that is a member of the family Herpesviridae. It can infect both warm- and cold-blooded vertebrates by contact between moist mucosal surfaces.
Human Leukocyte Antigen (HLA): Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.
Humoral: Pertaining to elements found in body fluids (for example, humoral immunity and neutralizing antibodies).
Hybridization Dot Blot (DNA or RNA): A technique for detecting, analyzing, and identifying protein; similar to the Western blot but without electrophoretic separation of proteins.
Immunoassay: Technique for identifying substances based on the use of antibodies.
Insertional Mutagenesis: A type of mutation that is caused by the insertion of nucleic acid into a host-cell chromosome. There are multiple possible negative consequences of such an event, including death of a cell if an essential gene is inactivated or predisposition to cancer if a tumor suppressor gene is inactivated.
Integration: Assimilation (insertion via covalent binding) of genetic material (DNA) into the chromosome of a recipient cell.
Intrabodies: Intracellular antibodies that are not secreted and that are designed to bind and inactivate target molecules inside cells.
In Vivo: Procedure performed in the living organism.
In Vitro: Procedure performed outside of the living organism. It may involve cells or tissues derived from the organisms.
Leukemia: Malignant neoplasm of the blood-forming tissues.
Lipoplex: A formulation of lipids and polymers and/or proteins.
Liposome: A spherical lipid bilayer enclosing an aqueous compartment.
Mock Run: A test run that deliberately omits some critical reagents.
Monoclonal Antibodies: Antibodies that are derived from a single cell clone.
Naked DNA: Isolated, purified, and uncomplexed DNA (no protein or lipid).
Oligonucleotide: A polymer consisting of a small number of nucleotides, usually 5 to 30.
Oncogenes: Genes associated with neoplastic proliferation (cancer) following a mutation or perturbation in their expression.
Oncogenic: Cancer-causing.
Packaging Cell Line: Cell line that produces proteins required for packaging and production of viral vectors in an active form but does not produce replication-competent virus. It complements at the protein level what the vector is lacking genetically.
Parvovirus: DNA viruses of the family Parvoviridae. Host range includes many vertebrate species. Small, linear chain, single-stranded DNA with terminal hairpin loops.
Plasmid: A small circular form of DNA that carries certain genes and is capable of replicating independently in a host cell.
Process Validation: Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.
Producer Cell Line: An established cell line used to produce virus vectors, often at a large scale.
Polymerase Chain Reaction (PCR): Technique to amplify a target DNA or RNA sequence of nucleotides by repeated cycles of polymerase-based copying, resulting in geometric increases in copy number.
Potency: A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.
Promoter: DNA sequence that is located at the front of a gene and controls gene expression. It is required for binding of RNA polymerase to initiate transcription.
Recombinant DNA: DNA produced by joining fragments of DNA from different sources by in vitro manipulations.
Replication-competent Virus: A virus that can complete an entire replication cycle without a need for a helper virus; an autonomously replicating virus.
Restriction Endonuclease: An endonuclease that recognizes a specific sequence of bases within double-stranded DNA.
Retrovirus: A virus that contains reverse transcriptase, which converts viral RNA into DNA that then integrates into the host cell in a form called a provirus.
Serum-Free: Refers to cell growth medium that lacks a serum component.
S Phase: Part of the cell cycle during which DNA replication occurs (*Synthesis Phase*).
Stem Cell: Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.
Suspension Culture: Cells capable of growth in suspension, not requiring substrate (attachment) on which to grow.
Transduction: Transfer and expression of genetic material into a cell by means of a virus or phage vector.
Transfection: Transfer of DNA into cells by physical means such as by calcium phosphate coprecipitation.
Transgene: Refers to the foreign genetic material delivered as part of a vector construct.

Vector: The agent (plasmid, virus, or liposome–protein or DNA–protein complex) used to introduce nucleic acid into a cell.
Viability: State of being alive and functional.
Virion: An elementary viral particle consisting of genetic material (nucleocapsid) and a protein covering.
Virus: Submicroscopic infectious agent that contains genetic information necessary for reproduction. It is an obligate intracellular parasite.
Western Blot: An electroblotting method in which proteins are transferred from a gel to a thin, rigid support (e.g., nitrocellulose membrane) and detected by binding radioactively labeled antibody or antibody coupled to an enzyme, allowing use of a precipitating chromogenic or chemiluminescent substrate.
Xenogeneic: From a different species.

APPENDIX

List of Relevant Regulatory References

Gene therapy products are regulated by the FDA as biologics, and therefore their manufacturing, testing, labeling, and other factors are subject to the requirements codified in CFR and FDA guidance documents (www.fda.gov). Additional guidance is provided in ICH guidelines (www.ich.org). Manufacturers of gene therapy products that seek markets outside the United States should refer to regulatory documents from relevant countries. Beyond *USP* chapters, the following list includes regulatory documents, as well as best practices for the development, manufacturing, quality control, and quality assurance of gene therapy products:

CFR

21 CFR 210
21 CFR 211
21 CFR 600s
21 CFR 610 Subpart G
21 CFR 801
21 CFR 820

FDA GUIDANCE DOCUMENTS

Guideline for the Determination of Residual Moisture in Dried Biological Products (January 1990)
Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy (March 1998)
Guidance for Industry: Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors (October 2000)
FDA Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production (October 2006)
Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) (April 2008)
Draft Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products (October 2008)

NATIONAL AND INTERNATIONAL REGULATORY DOCUMENTS

Q5A(R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin
ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products
ICH Q5D: Quality of Biotechnological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products
ICH Q6B Specification: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
ICH Q2 (R1): Validation of Analytical Procedures: Text and Methodology
NIH Guidelines for Research Involving Recombinant DNA Molecules

〈1048〉 QUALITY OF BIOTECHNOLOGICAL PRODUCTS: ANALYSIS OF THE EXPRESSION CONSTRUCT IN CELLS USED FOR PRODUCTION OF r-DNA DERIVED PROTEIN PRODUCTS[1]

I. INTRODUCTION

This document presents guidance regarding the characterization of the expression construct for the production of recombinant DNA (r-DNA) protein products in eukaryotic and prokaryotic cells. The document is intended to describe the types of information that are considered valuable in assessing the structure of the expression construct used to produce r-DNA derived proteins. The document is not intended to cover the entire quality aspect of r-DNA derived medicinal products.

The expression construct is defined as the expression vector containing the coding sequence of the recombinant protein. Segments of the expression construct should be analyzed using nucleic acid techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the final product. Analysis of the expression construct at the nucleic acid level should be considered as part of the overall evaluation of quality, taking into account that this testing only evaluates the coding sequence of a recombinant gene and not the translational fidelity nor other characteristics of the recombinant protein, such as secondary structure, tertiary structure, and posttranslational modifications.

II. RATIONALE FOR THE ANALYSIS OF THE EXPRESSION CONSTRUCT

The purpose of analyzing the expression construct is to establish that the correct coding sequence of the product has been incorporated into the host cell and is maintained during culture to the end of production. The genetic sequence of recombinant proteins produced in living cells can undergo mutations that could alter the properties of the protein with potential adverse consequences to patients. No single experimental approach can be expected to detect all possible modifications to a protein. Protein analytical techniques can be used to assess the amino acid sequence of the protein and structural features of the expressed protein due to posttranslational modifications such as proteolytic processing, glycosylation, phosphorylation, and acetylation. Data from nucleic acid analysis may be useful because protein analytical methods may not detect all changes in protein structure resulting from mutations in the sequence coding for the recombinant protein. The relative importance of nucleic acid analysis and protein analysis will vary from product to product.

Nucleic acid analysis can be used to verify the coding sequence and the physical state of the expression construct. The nucleic acid analysis is performed to ensure that the expressed protein will have the correct amino acid sequence, but is not intended to detect low levels of variant sequences. Where the production cells have multiple integrated copies of the expression construct, not all of which may be transcriptionally active, examination of the transcription product itself by analysis of m-RNA or c-DNA may be more appropriate than analysis of genomic DNA. Analytical approaches that examine a bulk population of nucleic acids, such as those performed on pooled clones or material amplified by the polymerase chain reaction, may be considered as an alternative to approaches that depend on selection of individual DNA clones. Other techniques could be considered that allow for rapid and sensitive confirmation of the sequence coding for the recombinant protein in the expression construct.

The following sections describe information that should be supplied regarding the characterization of the expression construct during the development and validation of the production system. Analytical methodologies should be validated for the intended purpose of confirmation of sequence. The validation documentation should, at a minimum, include estimates of the limits of detection for variant sequences. This should be performed for either nucleic acid or protein sequencing methods. The philosophy and recommendations for analysis expressed in this document should be reviewed periodically to take advantage of new advances in technology and scientific information.

[1] This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 29, 1995. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on February 23, 1996 (61 FR 7006) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on the production of r-DNA derived protein products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the "Regulatory Guidance" section).

III. CHARACTERIZATION OF THE EXPRESSION SYSTEM

A. Expression Construct and Cell Clone Used to Develop the Master Cell Bank (MCB)

The manufacturer should describe the origin of the nucleotide sequence coding for the protein. This should include identification and source of the cell from which the nucleotide sequence was originally obtained. Methods used to prepare the DNA coding for the protein should be described.

The steps in the assembly of the expression construct should be described in detail. This description should include the source and function of the component parts of the expression construct, e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether or not the protein is being synthesized as a fusion protein. A detailed component map and a complete annotated sequence of the plasmid should be given, indicating those regions that have been sequenced during the construction and those taken from the literature. Other expressed proteins encoded by the plasmid should be indicated. The nucleotide sequence of the coding region of the gene of interest and associated flanking regions that are inserted into the vector, up to and including the junctions of insertion, should be determined by DNA sequencing of the construct.

A description of the method of transfer of the expression construct into the host cell should be provided. In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail.

B. Cell Bank System

Production of the recombinant protein should be based on well-defined MCB and Working Cell Banks (WCB). A cell bank is a collection of ampules of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells. The MCB is generally derived from the selected cell clone containing the expression construct. The WCB is derived by expansion of one or more ampules of the MCB. The cell line history and production of the cell banks should be described in detail, including methods and reagents used during culture, in vitro cell age, and storage conditions. All cell banks should be characterized for relevant phenotypic and genotypic markers, which could include the expression of the recombinant protein or presence of the expression construct.

The expression construct in the MCB should be analyzed as described below. If the testing cannot be carried out on the MCB, it should be carried out on each WCB.

Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for copy number, for insertions or deletions, and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells retaining the expression construct should be determined.

The protein coding sequence for the recombinant protein product of the expression construct should be verified. For extrachromosomal expression systems, the expression construct should be isolated and the nucleotide sequence encoding the product should be verified without further cloning. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies. Alternatively, the nucleic acid sequence encoding the product could be verified by techniques such as sequencing of pooled c-DNA clones or material amplified by the polymerase chain reaction. The nucleic acid sequence should be identical, within the limits of detection of the methodology, to that determined for the expression construct as described in section III.A., and should correspond to that expected for the protein sequence.

C. Limit for In Vitro Cell Age for Production

The limit for in vitro cell age for production should be based on data derived from production cells expanded under pilot plant-scale or full-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could be used to prepare the production cells with appropriate justification.

The expression construct of the production cells should be analyzed once for the MCB as described in section III.B., except that the protein coding sequence of the expression construct in the production cells could be verified by either nucleic acid testing or analysis of the final protein product. Increases in the defined limit for in vitro cell age for production should be supported by data from cells that have been expanded to an in vitro cell age that is equal to or greater than the new limit for in vitro cell age.

IV. CONCLUSION

The characterization of the expression construct and the final purified protein are both important to ensure the consistent production of a r-DNA derived product. As described above, analytical data derived from both nucleic acid analysis and evaluation of the final purified protein should be evaluated to ensure the quality of a recombinant protein product.

GLOSSARY

Expression Construct: The expression vector that contains the coding sequence of the recombinant protein and the elements necessary for its expression.
Flanking Control Regions: Noncoding nucleotide sequences that are adjacent to the 5′ and 3′ end of the coding sequence of the product that contain important elements that affect the transcription, translation, or stability of the coding sequence. These regions include, e.g., promoter, enhancer, and splicing sequences, and do not include origins of replication and antibiotic resistance genes.
Integration Site: The site where one or more copies of the expression construct is integrated into the host cell genome.
In Vitro Cell Age: Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time in culture, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.
Master Cell Bank (MCB): An aliquot of a single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the MCB unless justified.
Pilot Plant Scale: The production of a recombinant protein by a procedure fully representative of and simulating that to be applied on a full commercial manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.
Relevant Genotypic and Phenotypic Markers: Those markers permitting the identification of the strain of the cell line that should include the expression of the recombinant protein or presence of the expression construct.
Working Cell Bank (WCB): The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

⟨1049⟩ QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS[1]

INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the product's potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

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[1] This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 20, 1995. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the *Federal Register* on July 10, 1996 (61 FR 36466) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on stability testing of biotechnological/biological products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the "Regulatory Guidance" section).

SCOPE OF THE ANNEX (2)

The guidance stated in this annex to "Stability Testing of New Drug Substances and Products" applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the "Glossary" in "Stability Testing of New Drug Substances and Products." However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

SELECTION OF BATCHES (4)

Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with acommitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those

long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable in vivo or in vitro quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, in vitro tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

STORAGE CONDITIONS (6)

Temperature (6.1)

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

Humidity (6.2)

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

Accelerated and Stress Conditions (6.3)

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in

determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

Light (6.4)

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

Container/Closure (6.5)

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

Stability after Reconstitution of Freeze-Dried Product (6.6)

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

TESTING FREQUENCY (7)

The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

GLOSSARY (10)

Conjugated Product: A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.
Degradation Product: A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products, some degradation products may be active.
Impurity: Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.
Intermediate: For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.
Manufacturing Scale Production: Manufacture at the scale typically encountered in a facility intended for product production for marketing.
Pilot-Plant Scale: The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

〈1050〉 VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect), and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document, the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterized cell banks. It covers products derived from in vitro cell culture, such as interferons, monoclonal antibodies, and recombinant deoxyribonucleic acid (DNA)-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown in vivo as ascites. In this latter case, special considerations apply and additional information on testing cells propagated in vivo is contained in *Appendix 1*. Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

(1) Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;

(2) Assessing the capacity of the production processes to clear infectious viruses;

(3) Testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to describe a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the MCB by several routes such as: (1) Derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

B. Adventitious Viruses That Could Be Introduced During Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: (1) Use of contaminated biological reagents such as animal serum components; (2) use of a virus for the induction of expression of specific genes encoding a desired protein; (3) use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used for Production

Table 1 shows examples of virus tests to be performed once only at various cell levels, including MCB, WCB, and cells at the limit of in vitro cell age used for production.

Table 1. Examples of Virus Tests to be Performed Once At Various Cell Levels

	MCB	WCB[1]	Cells at the Limit[2]
Tests for Retroviruses and Other Endogenous Viruses			
Infectivity	+	−	+
Electron microscopy[3]	+[3]	−	+[3]
Reverse transcriptase[4]	+[4]	−	+[4]
Other virus-specific tests[5]	as appropriate[5]	−	as appropriate[5]
Tests for Nonendogenous or Adventitious Viruses			
In vitro Assays	+	−[6]	+
In vivo Assays	+	−[6]	+
Antibody production tests[7]	+[7]	−	−
Other virus-specific tests[8]	+[8]	−	−

[1] See text—section III.A.2.
[2] Cells at the limit: Cells at the limit of in vitro cell age used for production (See text—section III.A.3.).
[3] May also detect other agents.
[4] Not necessary if positive by retrovirus infectivity test.
[5] As appropriate for cell lines which are known to have been infected by such agents.
[6] For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB's subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells at the limit of in vitro cell age.
[7] e.g., MAP, RAP, HAP—usually applicable for rodent cell lines.
[8] e.g., tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate.

1. MASTER CELL BANK

Extensive screening for both endogenous and nonendogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or nonhuman primate in origin, tests should be performed in order to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for nonendogenous viruses should include in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

2. WORKING CELL BANK

Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB. When appropriate nonendogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of in vitro cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

3. CELLS AT THE LIMIT OF IN VITRO CELL AGE USED FOR PRODUCTION

The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. The performance of suitable tests (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. *Table 2* outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can

be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or nonhuman primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus

Test	Test Article	Detection Capability	Detection Limitation
Antibody production	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system
In vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
In vitro virus screen for:		Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
1. Cell bank characterization	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
2. Production screen	2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM on:		Virus and virus-like particles	Qualitative assay with assessment of identity
1. Cell substrate	1. Viable cells		
2. Cell culture supernatant	2. Cell-free culture supernatant		
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system
Cocultivation	Viable cells	Infectious retroviruses	RV failing to replicate
1. Infectivity endpoint			1. See above under RV infectivity
2. TEM endpoint			2. See above under TEM[1]
3. RT endpoint			3. See above under RT
PCR (Polymerase chain reaction)	Cells, culture fluid and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious.

[1] In addition, difficult to distinguish test article from indicator cells.

1. TESTS FOR RETROVIRUSES

For the MCB and for cells cultured up to or beyond the limit of in vitro cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Induction studies have not been found to be useful.

2. IN VITRO ASSAYS

In vitro tests are carried out by the inoculation of a test article (see *Table 2*) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

3. IN VIVO ASSAYS

A test article (see *Table 2*) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

4. ANTIBODY PRODUCTION TESTS

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see *Table 2*) into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in *Table 3*.

Table 3. Virus Detected in Antibody Production Tests

MAP	HAP	RAP
Ectromelia Virus[2,3]	Lymphocytic Choriomeningitis Virus (LCM)[1,3]	Hantaan Virus[1,3]
Hantaan Virus[1,3]	Pneumonia Virus of Mice (PVM)[2,3]	Kilham Rat Virus (KRV)[2,3]
K Virus[2]	Reovirus Type 3 (Reo3)[1,3]	Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]
Lactic Dehydrogenase Virus (LDM)[1,3]	Sendai Virus[1,3]	Pneumonia Virus of Mice (PVM)[2,3]
Lymphocytic Choriomeningitis Virus (LCM)[1,3]	SV5	Rat Coronavirus (RCV)[2]
Minute Virus of Mice[2,3]		Reovirus Type 3 (Reo3)[1,3]
Mouse Adenovirus (MAV)[2,3]		Sendai Virus[1,3]
Mouse Cytomegalovirus (MCMV)[2,3]		Sialoacryoadenitis Virus (SDAV)[2]
Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]		Toolan Virus (HI)[2,3]
Mouse Hepatitis Virus (MHV)[2]		
Mouse Rotavirus (EDIM)[2,3]		
Pneumonia Virus of Mice (PVM)[2,3]		
Polyoma Virus[2]		
Reovirus Type 3 (Reo3)[1,3]		
Sendai Virus[1,3]		
Thymic Virus[2]		

[1] Viruses for which there is evidence of capacity for infecting humans or primates.
[2] Viruses for which there is no evidence of capacity for infecting humans.
[3] Virus capable of replicating in vitro in cells of human or primate origin.

C. Acceptability of Cell Lines

It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, the action plan recommended for manufacture is described in section V. of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least three lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. In vitro screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterizing the clearance of nonspecific "model" viruses (described later). Definitions of "relevant," specific, and nonspecific "model" viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. When a manufacturing process is characterized for robustness of clearance using nonspecific "model" viruses, particular attention should be paid to nonenveloped viruses in the study design. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described in section VI. below.

Table 4 presents an example of an action plan in terms of process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterization of clearance using nonspecific "model" viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the regulatory authorities. With Cases C, D, and E, it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk

	Case A	Case B	Case C[2]	Case D[2]	Case E[2]
Status					
Presence of virus[1]	–	–	+	+	(+)[3]
Virus-like particles[1]	–	–	–	–	(+)[3]
Retrovirus-like particles[1]	–	+	–	–	(+)[3]
Virus identified	not applicable	+	+	+	–
Virus pathogenic for humans	not applicable	–[4]	–[4]	+	unknown
Action					
Process characterization of viral clearance using nonspecific "model" viruses	yes[5]	yes[5]	yes[5]	yes[5]	yes[7]

[1] Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.

[2] The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.

[3] Virus has been observed by either direct or indirect methods.

[4] Believed to be nonpathogenic.

[5] Characterization of clearance using nonspecific "model" viruses should be performed.

[6] Process evaluation for "relevant" viruses or specific "model" viruses should be performed.

[7] See text under Case E.

[8] The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk *(Continued)*

	Case A	Case B	Case C[2]	Case D[2]	Case E[2]
Process evaluation of viral clearance using "relevant" or specific "model" viruses	no	yes[6]	yes[6]	yes[6]	yes[7]
Test for virus in purified bulk	not applicable	yes[8]	yes[8]	yes[8]	yes[8]

[1] Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.
[2] The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.
[3] Virus has been observed by either direct or indirect methods.
[4] Believed to be nonpathogenic.
[5] Characterization of clearance using nonspecific "model" viruses should be performed.
[6] Process evaluation for "relevant" viruses or specific "model" viruses should be performed.
[7] See text under Case E.
[8] The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

Case A

Where no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or in the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific "model" viruses as previously stated.

Case B

Where only a rodent retrovirus (or a retrovirus-like particle that is believed to be nonpathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific "model" virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as Chinese hamster ovary (CHO), C127, baby hamster kidney (BHK), and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk. Studies with nonspecific "model" viruses, as in Case A, are appropriate.

Case C

When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans (such as those identified by footnote 2 in *Table 3*, except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, "relevant" or specific "model" viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or "relevant" or specific "model") viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case D

Where a known human pathogen, such as those indicated by footnote 1 in *Table 3*, is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, "relevant" and/or specific "model" viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case E

When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.

VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterization of due virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterized cell lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see section VI.B.5.).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale, which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses exhibiting a range of biochemical and biophysical properties that are not known or expected to be present is to characterize the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C.). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value need not be achieved.

A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterization study and the guidance provided in this document.

1. "RELEVANT" VIRUSES AND "MODEL" VIRUSES

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: "Relevant" viruses, specific "model" viruses, and nonspecific "model" viruses.

"Relevant" viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a "relevant" virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown in vitro to sufficiently high titers), a specific "model" virus should be used as a substitute. An appropriate specific "model" virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

General Chapters

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in *Appendix 2* and *Table A-1*.

2. OTHER CONSIDERATIONS

Additional points to be considered are as follows:

(a) Viruses which can be grown to high titer are desirable, although this may not always be possible.

(b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.

(c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

General Chapters

B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

1. FACILITY AND STAFF

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

2. SCALED-DOWN PRODUCTION SYSTEM

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations that cannot be avoided should be discussed with regard to their influence on the results.

3. ANALYSIS OF STEP-WISE ELIMINATION OF VIRUS

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (*Appendix 4*).

4. DETERMINING PHYSICAL REMOVAL VERSUS INACTIVATION

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

5. INACTIVATION ASSESSMENT

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast "phase 1" and a slow "phase 2." The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a "relevant" virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific "model" viruses are used or when specific "model" viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

6. FUNCTION AND REGENERATION OF COLUMNS

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

7. SPECIFIC PRECAUTIONS

(a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.

(b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.

(c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as the dilution, concentration, filtration or storage of samples before titration.

(d) The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.

(e) Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.

(f) Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.

(g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.

(h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.

(i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies; Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See *Appendix 5* for calculation of virus reduction factors and *Appendix 6* for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:

(i) The appropriateness of the test viruses used;
(ii) The design of the clearance studies;
(iii) The log reduction achieved;
(iv) The time dependence of inactivation;
(v) The potential effects of variation in process parameters on virus inactivation/removal;
(vi) The limits of assay sensitivities;
(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well-designed separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 $\log_{10}$ or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.

2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 $\log_{10}$), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.

4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 $\log_{10}$ infectious units per milliliter (mL) by a factor of 8 $\log_{10}$ leaves zero $\log_{10}$ per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.

5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.

6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see *Appendix 3*).

F. Reevaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

A. Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;

B. Assessment of risk by determination of the human tropism of the contaminants;

C. Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;

D. Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and

E. Performance of studies which assess virus inactivation and removal.

General Chapters

GLOSSARY

Adventitious Virus: See *Virus.*

Cell Substrate: Cells used to manufacture product.

Endogenous Virus: See *Virus.*

Inactivation: Reduction of virus infectivity caused by chemical or physical modification.

In Vitro Cell Age: A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Master Cell Bank (MCB): An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the original MCB, unless justified.

Minimum Exposure Time: The shortest period for which a treatment step will be maintained.

Nonendogenous Virus: See *Virus.*

Process Characterization of Viral Clearance: Viral clearance studies in which nonspecific "model" viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

Process Evaluation Studies of Viral Clearance: Viral clearance studies in which "relevant" and/or specific "model" viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

Production Cells: Cell substrate used to manufacture product.

Unprocessed Bulk: One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

Virus: Intracellularly replicating infectious agents that are potentially pathogenic, possess only a single type of nucleic acid (either ribonucleic acid (RNA) or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

Adventitious Virus: Unintentionally introduced contaminant virus.

Endogenous Virus: Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

Nonendogenous Virus: Virus from external sources present in the MCB.
Nonspecific Model Virus: A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the purification process.
Relevant Virus: Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.
Specific Model Virus: Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.
Viral Clearance: Elimination of target virus by removal of viral particles or inactivation of viral infectivity.
Virus-like Particles: Structures visible by electron microscopy which morphologically appear to be related to known viruses.
Virus Removal: Physical separation of virus particles from the intended product.
Working Cell Bank (WCB): The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

APPENDICES

Appendix 1: Products Derived from Characterized Cell Banks Which Were Subsequently Grown In Vivo

For products manufactured from fluids harvested from animals inoculated with cells from characterized banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in *Table 3*, should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum, and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in section IV. of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as in vivo testing in adult and suckling mice.

Appendix 2: The Choice of Viruses for Viral Clearance Studies

A. Examples of Useful *"Model" Viruses:*

1. Nonspecific "model" viruses representing a range of physico-chemical structures:
• SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, nonenveloped viruses;
• a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
• a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.
These viruses are examples only and their use is not mandatory.
2. For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

B. Examples of Viruses That Have Been Used in Viral Clearance Studies

Several viruses that have been used in viral clearance studies are listed in *Table A-1*. However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers are invited to consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

Table A-1. Examples of Viruses Which Have Been Used in Viral Clearance Studies

Virus	Family	Genus	Natural Host	Genome	Env	Size (nm)	Shape	Resistance[1]
Vesicular Stomatitis Virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70 × 150	Bullet	Low
Parainfluenza Virus	Paramyxo	Paramyxovirus	Various	RNA	yes	100–200	Pleo/Spher	Low
MuL V	Retro	Type C oncovirus	Mouse	RNA	yes	80–110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60–70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50–70	Pleo/Spher	Low
Pseudo-rabies Virus	Herpes		Swine	DNA	yes	120–200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25–30	Icosahedral	Med
Encephalomyocarditis Virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	no	25–30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	RNA	no	60–80	Spherical	Med
SV 40	Papova	Polyomavirus	Monkey	DNA	no	40–50	Icosahedral	Very high
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine	DNA	no	18–24	Icosahedral	Very high

[1] Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory.

Appendix 3: Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately $0.5 \log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses. If the 95 percent confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95 percent confidence limits for the reduction factor are

$$\pm\sqrt{S^2 + a^2}$$

Appendix 4: Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$$p = ((V-v)/V)^n$$

where V (L) is the overall volume of the material to be tested; v (L) is the volume of the sample; and n is the absolute number of infectious particles statistically distributed in V.

If V >> v, this equation can be approximated by the Poisson distribution:

$$p = e^{-cv}$$

where c is the concentration of infectious particles per L.

$$\text{or, } c = \ln p/{-v}$$

As an example, if a sample volume of 1 mL is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

c	10	10	1,000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

Appendix 5: Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material: vol v′; titer $10^{a'}$;
virus load: $(v')(10^{a'})$,
Final material: vol v″; titer $10^{a''}$;
virus load: $(v'')(10^{a''})$,
the individual reduction factors Ri are calculated according to
$10^{Ri} = (v')(10^{a'}) / (v'')(10^{a''})$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

Appendix 6: Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.
Example:
I. Assumptions
Measured or estimated concentration of virus in cell culture harvest = 10^6/mL
Calculated viral clearance factor => 10^{15}
Volume of culture harvest needed to make a dose of product = 1 L (10^3 mL)
II. Calculation of Estimated Particles/Dose

$$\frac{\left(10^6 \text{ virus units/mL}\right) \times \left(10^3 \text{ mL/dose}\right)}{\text{Clearance factor} > 10^{15}}$$

$$= \frac{10^9 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}}$$

$$= < 10^{-6} \text{ particles/dose}$$

Therefore, less than one particle per million doses would be expected.

〈1050.1〉 DESIGN, EVALUATION, AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

INTRODUCTION

This chapter is a companion document to *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* 〈1050〉, which was adapted, essentially unchanged, from the International Conference on Harmonization Q5A. This chapter provides users with practical guidance regarding the design, evaluation, and characterization of viral clearance procedures. The chapter scope is the same as that described in 〈1050〉 and covers biotechnology products for human use that are derived from cell lines of human or animal origin. Viral clearance studies performed according to the principles outlined in this chapter will provide meaningful data about the ability of the overall production and purification processes to remove or inactivate a broad spectrum of viral types that may affect the safety of biotechnology-derived products. [NOTE—The *Glossary* contains definitions of terms used in this chapter that are not already defined in 〈1050〉.]

The regulations for licensing biotechnology products stipulate that cell banks, biologically derived raw materials, and bulk harvest must be controlled and tested for viral safety; however, many of the viral detection approaches that can be used have inherent limitations. In addition, certain detection methods may be so specific for a particular virus that they may fail to detect viral variants (e.g., noncytopathic strains, or non-laboratory-adapted wild-type strains, or mutated variants). Some newly emerging viruses may be missed. Finally, detection methods typically are limited by their sensitivities, and infectious viruses at a low titer may go undetected. Viral contamination of manufacturing processes can come from many sources, including cell substrates and raw materials of biological origin (such as cell culture supplements and other production raw materials), and contamination during production. In addition to the required viral safety testing of these materials, manufacturers must evaluate the effectiveness of downstream purification process steps, which together must remove or inactivate any viruses potentially present (see 〈1050〉). USP encourages readers to maintain and keep current best practices in the field by reviewing current peer-reviewed publications, regulatory guidances, and key opinion leaders and organizations.

As suggested in the U.S. Food and Drug Administration "Points to Consider" documents (*Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use* and *Draft Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*), as well as 〈1050〉, a multi-step approach is typically used to ensure viral safety. Clearance studies can generally be considered generic or modular. As described in the FDA's *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use*, a generic clearance study is one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model monoclonal antibody (mAb) in which the data may be extrapolated to other mAbs following the same purification and virus removal/inactivation scheme as the model mAb. A modular clearance study demonstrates virus removal or inactivation of individual steps during the purification process (column chromatography, filtration, pasteurization, solvent/detergent, low pH, and others) so that each module in the purification scheme may be studied independently of the other modules. If necessary, an alternative model mAb can be used to demonstrate viral clearance in different modules. Identical clearance modules can be extrapolated to the product mAb. This chapter complements these documents by providing users strategies to perform and assess viral clearance.

GOALS AND PRINCIPLES OF VIRAL CLEARANCE STUDIES

A viral clearance study should evaluate the ability of the overall purification process to remove or inactivate a broad spectrum of virus types, including viruses that are known to contaminate or have the potential to contaminate the raw materials, and those that can be introduced during manufacturing. Viral clearance studies typically involve substudies performed on specific and suitable individual steps of the manufacturing and purification process. The studies should be performed in a manner that generates quantitative data, allowing for identification of effective clearance steps and estimation of viral reduction factors (VRFs, also known as log reduction factors, LRF, or log reduction values, LRV). The VRFs of an individual clearance step represent the ratio of the viral load in the pretreatment material (used to challenge the clearance step) to the viral load in the post-treatment material. The VRFs derived from specific process steps are used to evaluate the overall capacity of the entire production process to remove or inactivate process-specific or nonspecific viruses.

One of the key goals of a manufacturing purification process is to achieve maximal viral clearance without compromising product quality. Critical attributes of strategic viral clearance steps in the manufacturing process must be characterized. In the context of viral clearance, robustness has two main components: 1) the ability of process steps to remove and/or inactivate viruses under worst-case conditions or over a wide operational range for parameters (e.g., temperature, protein concentration, pressure, flow rate, conductivity, and pH), and 2) the ability of process steps to consistently remove or inactivate nonspecific viruses that possess a broad spectrum of physical and chemical resistance characteristics (e.g., pH, heat, solvent/detergent treatment, or filtration). A validated process that provides robust viral clearance should establish the VRF achievable for a panel of "relevant" or "model" viruses (see next section for definitions). The validated process also should demonstrate robustness of dedicated inactivation or removal steps for which critical operational parameters that could affect viral clearance are well estab-

lished. Therefore, a demonstration of viral clearance robustness provides confidence that the manufacturing process can remove or inactivate potential viral contaminants.

Viral clearance study protocols begin with an overall action plan and design of experiments. Chapter ⟨1050⟩ (see *Section V* and *Table 4*) describes five major action plans (*Cases A–E*) and can serve as a guide. Cell substrates categorized in *Cases C, D,* and *E* represent special cases, and manufacturers who use these substrates should discuss study design with the applicable regulatory authorities. Unique issues for each production process must be considered on a case-by-case basis. These issues may relate to the starting materials, production process, product, and intended use of the product. The overall action plan dictates the choice of viruses used in the viral clearance study, i.e., the study must include relevant, specific, and nonspecific model viruses. Further, the viral clearance study protocol may include the following elements: descriptions of the study facility and staff responsible for executing the study, the scaled-down purification models, justification of the appropriateness of the scaled-down model, and the study design. The study report may include these elements, as well as a stepwise analysis of calculated VRFs and the overall viral clearance capacity of the purification process. Viral clearance studies should be conducted in a well-documented, controlled manner that complies with current regulatory requirements.

CONSIDERATIONS FOR PERFORMANCE OF VIRAL CLEARANCE STUDIES

Selection of Viruses

The choice and number of viruses that may be used in a viral clearance study are dictated by the nature and origin of the production cell line, as well as the nature and origin of the animal-derived materials used in production and purification. In general, at least two viruses, one enveloped (typically a retrovirus, e.g., MuLV) and one nonenveloped (preferably a parvovirus, e.g., MVM), are used in the early clinical phases of product development, but three or more viruses may be used to generate data for registration-enabling studies. At least two orthogonal virus removal/inactivation steps (steps with different mechanisms of clearance) should be evaluated per virus. The reproducibility of an effective step should be assessed by performing at least two independent experiments, or reproducibility should be supported by the process development history (or experiences). Model viruses for process evaluation and process characterization studies should be similar to the virus that may contaminate the product; however, other viruses with a wide range of physical and chemical characteristics should also be examined. The latter is important for showing that the purification process is capable of inactivating or removing a wide variety of viruses, including newly emerging viruses (e.g., vesivirus 2117, circoviruses, or newly discovered animal and human parvoviruses) or unexpected contaminants (e.g., BVDV, MVM, epizootic hemorrhagic disease virus, and Cache valley virus). The manufacturer should justify the choice of viruses in accordance with the aims of the process evaluation and process characterization studies and the guidance provided in this document, as well as relevant regulatory guidelines.

Viruses used in clearance studies fall into three categories: relevant viruses, specific model viruses, and nonspecific model viruses. "Relevant viruses" are viruses used in process evaluation studies, which are either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. When a relevant virus is not available or when it is not well adapted for this purpose (e.g., it cannot be grown in vitro to a sufficiently high titer or cannot be detected using cell-based viral titration endpoints required for assessing inactivation), a "specific model virus" can be used as a substitute. An appropriate specific model virus may be a virus that is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus. For example, cell lines derived from rodents usually contain endogenous retroviral particles or retroviral-like particles that can be infectious or noninfectious. The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses present in products obtained from such cells should be determined. In the case of rodent-derived cells, one can use a murine leukemia virus as a model virus. Manufacturers using human-derived B-lymphocyte cells immortalized by infection with Epstein–Barr virus should demonstrate the ability of the manufacturing process to clear a herpesvirus. In this instance, pseudorabies virus or another herpesvirus can be used as a specific model virus.

Finally, when the goal is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general (i.e., to characterize the robustness of the clearance process), viral clearance studies can be performed with a panel of "nonspecific model viruses" that have a wide range of physical and chemical characteristics. Data obtained from studies with relevant or specific model viruses also can contribute to this assessment. It is not necessary to test all types of viruses; preference should be given to viruses with diverse physical and chemical properties that would be representative of a wide range of viral families. The results obtained for such viruses provide useful information about the ability of the production process to remove or inactivate viruses in general. *Table 1* gives examples of model viruses that represent a range of physicochemical properties for use in viral clearance studies, including those examples already described in ⟨1050⟩ (see *Table A-1*). Additional points to consider during viral selection are: 1) ability to grow the virus of interest to a sufficiently high titer, 2) ability to create stocks with minimal aggregates, 3) availability of a qualified/validated assay system for detection of the selected virus, 4) the selected viruses are not likely to pose a health hazard to personnel performing the study or the environment, and 5) potential to address new and emerging viruses.

Table 1. Examples of Viruses Used in Viral Clearance Studies for Biotechnology Products Derived from Cell Cultures

Virus	Family	Genus	Natural Host	Genome	Enveloped	Size (nm)	Shape	Resistance[a]
Adenovirus 5	Adeno	Mastadeno-virus	Human	DNA	No	70–90	Spherical	High
BVDV	Flavi	Pestivirus	Bovine	RNA	Yes	50–70	Pleomorphic/ spherical	Low
Cache valley virus	Bunya	Bunyavirus	Bovine, ovine	RNA	Yes	80–120	Spherical	Low
Encephalomyocarditis virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	No	25–30	Icosahedral	Medium
Feline calicivirus	Calici	Vesivirus	Feline	RNA	No	35–40	Icosahedral	High
Herpes simplex 1	Herpes	Alpha-herpesvirus	Human	DNA	Yes	120–200	Spherical	Medium
MuLV	Retro	Type C oncovirus	Mouse	RNA	Yes	80–110	Spherical	Low
Parainfluenza virus	Paramyxo	Paramyxovirus	Various	RNA	Yes	100–200	Pleomorphic/ spherical	Low
Parvoviruses (MVM, PPV, CPV, BPV)	Parvo	Parvovirus	Murine, porcine, canine, bovine	DNA	No	18–26	Icosahedral	Very High
Poliovirus sabin type 1	Picorna	Enterovirus	Human	RNA	No	25–30	Icosahedral	Medium
Porcine circovirus (PCV)	Circo	Circovirus	Porcine	DNA	No	15–20	Icosahedral	High
Pseudorabies virus	Herpes	—	Swine	DNA	Yes	120–200	Spherical	Medium
Reovirus 3	Reo	Orthoreovirus	Various	RNA	No	60–80	Spherical	Medium
Simian virus 5	Paramyxo	Rubulavirus	Simian	RNA	Yes	150–300	Pleomorphic/ spherical	Low
Sindbis virus	Toga	Alphavirus	Human	RNA	Yes	60–70	Spherical	Low
SV40	Papova	Polyomavirus	Monkey	DNA	No	40–50	Icosahedral	Very high
Vesicular stomatitis virus	Rhabdo	Vesiculovirus	Equine, bovine	RNA	Yes	70 × 150	Bullet	Low
West Nile virus	Flavi	Flavivirus	Avian	RNA	Yes	40–70	Pleomorphic/ spherical	Medium

[a] Resistance to physicochemical treatments based on studies of purification processes. Resistance is relative to the specific treatment, and it is used in the context of understanding the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only, their use is not mandatory, and the table is not exhaustive.

Process Clearance Capability

The viral clearance capability of a process is typically expressed as the logarithmic value of the virus reduction factor evaluated by the entire process. Although there are no specific requirements for the clearance capability that a process must achieve, the nature and origin of the cell type, raw materials used in production, as well as other factors may influence the need for greater or lower levels of viral clearance. For example, the target retroviral clearance capability for a CHO and NS0 cell manufacturing process could be 4–6 LRF of total-process retroviral clearance above the level found in the maximum therapeutic dose, based on the retroviral-like particle titer in the production bioreactor material. Minimum clearance capability for other types of viruses might include >4 LRF of clearance for additional enveloped viruses and >4 LRF of clearance for nonenveloped viruses. Specific clearance targets must be established and justified on a case-by-case basis. In general, for a single virus clearance step to be considered effective, 4 LRF or more of clearance must be demonstrated. In contrast, a clearance step demonstrating 1–3 LRF is considered a supportive step. The titer of the virus input and output for any given process step evaluated should include 95% confidence ($\alpha = 0.05$). A step can be supportive for some viruses and effective for others. When possible and as process knowledge allows, the analyst should perform viral clearance studies under expected worst-case conditions for the parameters defined as critical for that step (e.g., pH, temperature, and flow rate). Sufficient numbers of downstream purification process steps should be identified and evaluated so that the required overall clearance target can be reached.

Downstream Processing Steps for Viral Clearance

Downstream processing steps should employ different (orthogonal) mechanisms of viral clearance because consideration of multiple steps of the same or similar clearance mechanisms may lead to overestimation of the overall viral reduction capability. In general, a purification process should include at least one robust viral inactivation step and/or one robust viral removal step. For every purification step assessed, the mechanism of viral clearance should be described as inactivation, removal, or a combination of both; if both, the primary clearance mechanism should be identified. For inactivation steps, clearance studies should be planned in such a way that they can determine the kinetics of viral inactivation. Ideally, a manufacturing process includes steps that are dedicated to viral clearance; that is, they are present in the production or purification process specifically for the removal or inactivation of viruses. Examples of dedicated steps include solvent and detergent treatment, low-pH inactivation, and virus-removal filtration.

Qualification and Scale-Down of Purification Steps

Viral clearance studies using infectious virus spikes are not conducted at production scale in the manufacturing suite because this could contaminate the suite with viruses. Such studies should be performed in a segregated facility equipped for virological work and staffed with personnel who have virology expertise and familiarity with the operation of a scaled-down purification process. Each scaled-down step of the purification process must be qualified, i.e., found to be comparable to the full-scale production process by all relevant, measurable criteria. Comparability should be demonstrated using representative raw materials and intermediates from production, and equipment with process parameters through appropriate scale-down principles. The outputs should be measured with the appropriate analytical methods and statistical analyses.

In many instances, the monitored parameters are the same as those analyzed during performance of the actual manufacturing steps. For chromatography, the parameters should be representative of the respective clinical or commercial-scale manufacturing: [e.g., the column bed height, residence time, linear flow rate, chromatographic matrix, buffer composition (including pH, conductivity, and operating temperature), and product pool concentrations and composition]. Scaled-down and manufacturing-scale chromatography systems should produce similar elution profiles and step yields, and final product analytical profile (e.g., SEC-HPLC and/or SDS-PAGE analyses). Similar considerations apply for other types of procedures. Any unavoidable differences between scaled-down and manufacturing-scale procedures should be investigated to determine their potential influence on the viral clearance results.

Selection of Sampling Points

Sampling points for assessing viral inactivation should include the starting materials (virus-spiked process solutions and appropriate controls) as well as samples taken at several time points during the inactivation process. This approach allows the analyst to monitor the kinetics of viral inactivation. Sampling points for virus-removal steps, such as chromatography and filtration, should include the feed-stream process solution that will be applied to the step, as well as the resulting process pool that is then processed in the subsequent steps. Chromatographic fractions (e.g., flow-through, washes, pre-peak, peak, post-peak, strip) of the mainstream, as well as pre- and post-mainstream samples, typically are evaluated in studies that support registration-enabling studies. Analyzing such side fractions might also be helpful in cases where the mechanism of partitioning is not understood. Sampling points are discussed further in the section *Performance of Viral Spiking Studies*.

Selection of Assays for Viral Quantitation

Various types of viral quantitation assays can be used. Examples include infectivity assays such as tissue-culture infectious-dose assays and plaque quantitation assays. In addition, quantitative polymerase chain reaction (qPCR) assays detect and quantify the nucleic acids of both infectious and noninfectious viruses. These assays are described in *Virology Test Methods* ⟨1237⟩, *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, and *Nucleic Acid Based Techniques—Amplification* ⟨1127⟩. When using nucleic acid-based techniques (NAT), it is important to assure that the virus spike and the spiked samples to be analyzed do not contain significant amounts of free viral nucleic acids. The amount of free nucleic acids can be reduced by nuclease pretreatment of samples. It is important to characterize the impact of nuclease pretreatment on the virus spike since it could have a negative impact on the virus spike. Quantitative/quantal infectivity assays should have adequate sensitivity and reproducibility, and should be performed with a sufficient number of replicates to ensure adequate statistical validity of the results. All assays should include appropriate system suitability controls. Virus titration assays used in support of viral clearance studies must be shown to be suitable for this purpose by means of qualification or validation.

Sample Matrix Effects on Viral Quantitation Assays

When a virus is cleared by inactivation or removal (e.g., filtration or column chromatography) methods, the analysts must perform preliminary cytotoxicity and viral interference analyses to determine if their viral quantitation assays are sensitive to matrix components present in their samples. These studies should be performed before the spiking studies to confirm that the results of the viral clearance spiking studies are actually due to viral clearance and not to other intrinsic or extrinsic factors yielding a false-negative result. For example, some process intermediates may be cytotoxic to the detector (indicator) cells or may interfere with virus detection by a particular assay system. The process solutions used in these studies should be representative of those from a full-scale good manufacturing practice (GMP) manufacturing run. The dilution of the process sample solution that does not cause cytotoxicity or interference is determined and the process solution is diluted accordingly before storage or testing samples from that manufacturing step in the virus-spiking studies.

In a cytotoxicity evaluation, the cells used in the viral quantitation assay are exposed to a series of dilutions of the sample matrix solution for that process step. Then the cells are assessed for viability and changes in morphology that could interfere with evaluation of the indicator cells for viral cytopathic effects or plaques and hence could have an impact on assay performance. The dilution of the process sample solution that does not cause cytotoxicity is determined, and the process solution is diluted accordingly before storage or testing samples from that manufacturing step in the virus-spiking studies.

The study involves the following steps:

1. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus inactivation or filtration load using appropriate cell culture media as the diluent
2. Inoculate the different dilutions of virus inactivation or filtration load alone onto the indicator cells that will be used for each virus titration
3. Include appropriate buffer or media and cell controls
4. Identify the dilution of the virus inactivation load that does not exhibit measurable detector cell toxicity attributable to the load matrix

Process solutions also can interfere with virus detection by the in vitro cell-based viral infectivity assay system, either by inactivating the virus itself or by altering the indicator cells in a way that delays or prevents occurrence of a productive viral infection. To assess a process solution for viral interference, analysts prepare dilutions of the process solution and spike them with a known quantity of virus. Each dilution is subsequently titrated in the quantitative/quantal viral infectivity assay. If the difference between the known titer and the titer determined for the spiked solution in the assay (the viral interference titer) differ by more than the predetermined assay variability (typically ±0.5 log), then viral interference is suspected.

To assess the inactivation step load for viral interference, analysts should follow these steps:

1. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus stock using appropriate cell culture media as the diluent. This will serve as the positive control for the virus titer
2. Perform serial dilutions (e.g., 10-fold, 5-fold, or 2-fold) of the virus stock using the virus inactivation load as the diluent [NOTE—Information from the cytotoxicity study will determine the design of this step and the next one.]
3. Inoculate the dilution series onto the indicator cells
4. Include assay suitability controls (positive and negative) in the test
5. Identify the dilution of the virus inactivation load that does not result in significant changes in virus stock titer compared to the positive control virus titer

If qPCR assays are used to quantitate the virus, each process solution should be tested for interference and recovery with the nucleic acid extraction method used, as well as for interference with the qPCR reaction itself. Each process solution must be diluted to a level that does not cause interference. Although diluting a process solution to achieve acceptable assay performance is a commonly used strategy, this reduces the maximum demonstrable VRF for the clearance step under evaluation.

Effects of Storing and Freezing Viral Clearance Samples

If process solutions are used within their established hold times, stability studies should be performed to better understand the effect on the clearance step of holding or storing the solutions, or on the virus (e.g., aggregation). For example, bacterial growth or precipitation of proteins in the solutions may occur, particularly after prolonged storage.

If the samples from the spiking study are stored frozen before virus quantitation, analysts should perform stability studies using the viral infectivity result as an endpoint. Ideally, this should be done before the viral spiking study by spiking dilutions of each process solution with a known amount of virus and then freezing each sample. After specified times in frozen storage, the samples are removed, and the titer of each sample dilution is determined. If the difference between the known titer and the titer determined from the spiked frozen solution is greater than the assay variability (typically ±0.5 log), this indicates that viral infectivity has been compromised by frozen storage.

Qualification of Virus Stocks and Effects on Processing Steps

The virus stocks used in viral clearance studies are critical reagents. Each virus stock should have a traceable, certified source with full documentation of the controlled production procedures. Virus stocks should meet predetermined criteria for identity, purity (e.g., sterility, mycoplasma, and adventitious virus testing), infectious titer, stability at freezing temperatures, and minimal viral aggregates. Analysts should minimize the number of passages from the master or working viral bank in order to reduce the chance of mutation. Purity of virus preparations should be taken into account in cases when virus preparation impurities may influence the performance of a certain unit operation (e.g., virus filtration).

During qualification, analysts should obtain an initial virus titer for each virus stock. Ideally, analysts should independently assay viral stocks for titer on separate days using different passage numbers of the indicator culture cells that were used in the viral quantification assay. The resulting titers should agree within the predetermined variability of the assay (typically the expectation is ±0.5 log), and if so, the analyst can average the titers to determine the certified titer of the stock.

It may be advisable to perform a mock spiking study before the true spiking study. This mock spiking study evaluates the effects of the spiking virus matrix on the process step that is under evaluation. Mock spiking studies are important for all steps that will be tested for viral clearance, but they are particularly important for those steps that will be evaluated for the clearance of virus from a stock that contains impurities or additives (e.g., stabilizing protein). Mock spiking studies are conducted by adding the viral suspension matrix (including all components except the virus) to the process solution at a virus-spike to feed-stream-solution (load) ratio of NMT 10% (v/v) and then executing the processing step. The mock-spiked step is monitored for expected performance, and if results are different than expected, then reducing the virus:load spiking ratio may be helpful. This adjusted virus-spike to feed-stream-solution (load) ratio would be used in performing the actual clearance study for the processing step under study.

Performance of Viral Spiking Studies

Viral clearance studies are usually conducted either at a site where both the spiking and viral quantitation assays are performed (e.g., a biosafety testing laboratory) or at two different sites: one where the spiking studies are done and a second where the samples are quantitated. Sponsors and analysts must gain knowledge and control factors that can influence each manipulation that is not part of the manufacturing process, for example, freezing and thawing (see *Effects of Storing and Freezing Viral Clearance Samples*) as well as shipping conditions.

Before initiating a clearance study, analysts should prepare a well-documented study design that clearly defines the following: the steps that will be tested, the sampling plan for each step, sample identification, sample handling and storage, sample shipment if required, critical operating parameters, process scale, rationale for worst-case conditions (if they have been established), and the appropriate controls for each step. At a minimum, the samples tested should include those used to determine: 1) the spiking virus titer, 2) the spiking virus titer after freeze–thaw (if applicable), 3) the virus titer in the production solution before processing, and 4) the virus titer after processing. The analyst may need to collect and assay additional samples, depending on the step under test and the phase of product development.

In a typical biomanufacturing process, viral clearance can be accomplished by virus inactivation (e.g., pH treatment, heat treatment, or solvent and detergent treatment) or by virus removal (e.g., filtration or column chromatography). *Figures 1A* and *1B* show examples of experimental designs for virus inactivation, *Figure 2* shows virus removal by filtration, and *Figure 3* shows virus removal by column chromatography. These examples are intended for general reference and do not represent every specific condition. In developing their study designs, sponsors should consider their own process conditions in order to plan their own specific steps.

The overall concept of viral clearance procedures can be expressed as follows:

Viral Clearance = Viral Inactivation + Viral Removal, where

Viral Removal = Removal by Filtration + Removal by Column Chromatography

VIRAL CLEARANCE BY VIRUS INACTIVATION

General considerations: Analysts should recognize that virus inactivation is not always a simple, first-order reaction. Typically, virus inactivation is complex and involves a fast initial phase followed by a slower phase. Thus, analysts should plan the inactivation process in such a way that samples are taken at different times in order to construct an inactivation time curve. Samples collected for inactivation studies should include the planned process time, and at a minimum, time zero; a time point or a suitable number of greater than zero but less than the minimum inactivating-agent exposure time; a time point equal to the minimum inactivating-agent exposure time; and in addition a time point beyond the minimum exposure time might be helpful in cases where there is a slow inactivation curve. Additional time points may be particularly important when there is no prior experience with virus inactivation kinetics.

When viral clearance by inactivation occurs, two scenarios generally are possible: 1) the inactivating agent is not present in the load material and inactivation is initiated by addition of the inactivating agent after virus spiking (*Figure 1A*); and 2) the load material already contains the inactivating agent before the virus spike, and inactivation is initiated by addition of the virus spike (*Figure 1B*). An example of the latter is a Protein A column eluate at low pH. Whenever possible, analysts should determine the initial virus load from the virus-spiked load material before they add an inactivating agent. However, this is not possible when the inactivating agent is already present in the load material at the start of the experiment. In these situations, the initial virus load can be calculated using the certified titer value of the spiking virus preparation and the virus:load spiking ratio. When viral inactivation occurs too rapidly to allow for plotting an inactivation time curve under normal process conditions, analysts should include appropriate controls to show that activity is indeed lost due to the inactivating agent present.

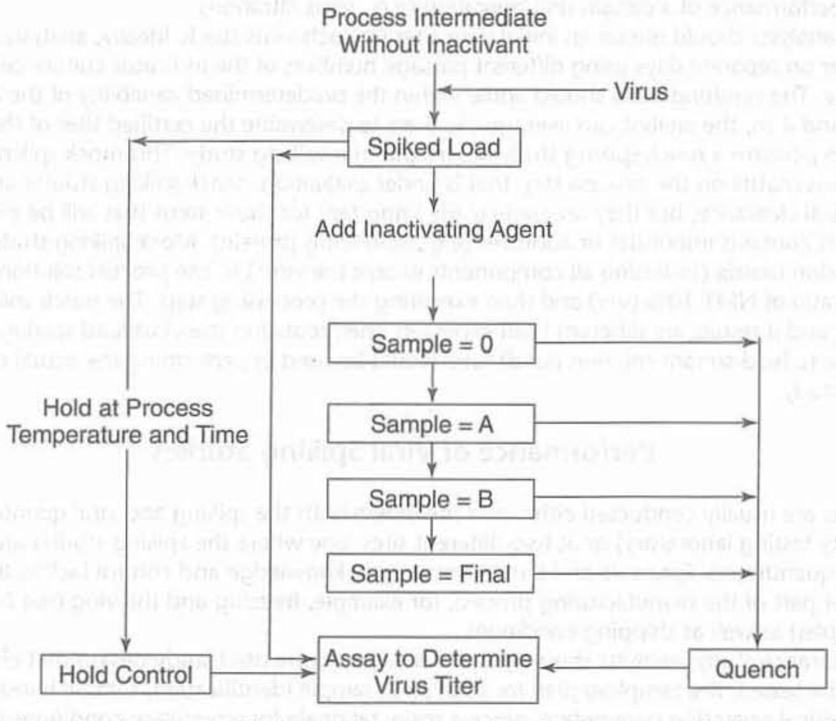


Figure 1A. Virus inactivation when load material does not contain an inactivating agent; the inactivating agent is added after viral spiking.

Study design: The general approach to the study design for assessing viral clearance by inactivation is outlined in *Figures 1A* and *1B*. In *Figure 1A*, the virus spike is added to the load material (process intermediate) before initiation of inactivation. The virus and load material are mixed well, and the spiked load and hold control samples are pulled. The spiked load should be tested immediately to determine virus titer. The actual titer of the spiked load (obtained experimentally) should be compared to the theoretical titer of the spiked load (obtained from the certified titer of the lot of virus used and the spiking ratio) to verify performance of virus detection assay and that the appropriate amount of virus was added to the load material. The hold control should be treated the same as the spiked load (i.e., prior to neutralization of the hold control, the sample should be pH adjusted in the same manner as the spiked load and then neutralized and spiked with virus). In addition, the hold control should remain at process temperature until the final sample in the experiment is pulled. The viral titer of the hold control then is assessed and compared to the actual titer of the spiked load to determine if any virus was inactivated in the presence of the load material over time and at the temperature of the inactivation study. This may be considered when determining the final VRF.

Inactivation should be initiated in a way that mimics the manufacturing process (e.g., with constant mixing) so that the inactivating agent (e.g., detergent, or acid utilized to lower pH) is mixed homogeneously into the solution as soon as possible.

This minimizes the presence of localized high concentrations of the inactivating agent. If heat is used for inactivation, the heat should be applied in a way that mimics manufacturing (e.g., the ramp to target temperature should be identical) and controls should be kept unheated. However, the exact reproduction of addition (mixing) of inactivating agents or reproduction of a heating ramp can be very difficult at down-scale. In such cases it is advised to spike the virus directly into the inactivation agent-containing (or heated) material and to follow inactivation kinetics as described in *Figure 1B*. For control of the added virus, virus is spiked into process material without the inactivating agent or into unheated material.

Worst-case process conditions (e.g., pH, time, and temperature) should be applied if they have been established. Time points (samples) are pulled and the reaction is immediately quenched (e.g., by neutralization, dilution, or immediate cooling of the sample) so that further viral inactivation cannot occur. This allows the analyst to determine the kinetics of virus inactivation and then construct a virus inactivation versus time curve. Large-volume sampling can be applied to maximize clearance values.

In *Figure 1B*, the load material contains the inactivating agent and thus the addition of the virus spike starts the inactivation. In this scenario, it may not be possible to neutralize the process intermediate solution before the virus-spiking study because large fluctuations in pH may cause the protein product to fall out of solution. Also, the spiked load and hold control samples cannot be pulled from the virus-spiked load material, thus the control samples are pulled from a media or buffer control that does not contain the inactivating agent.

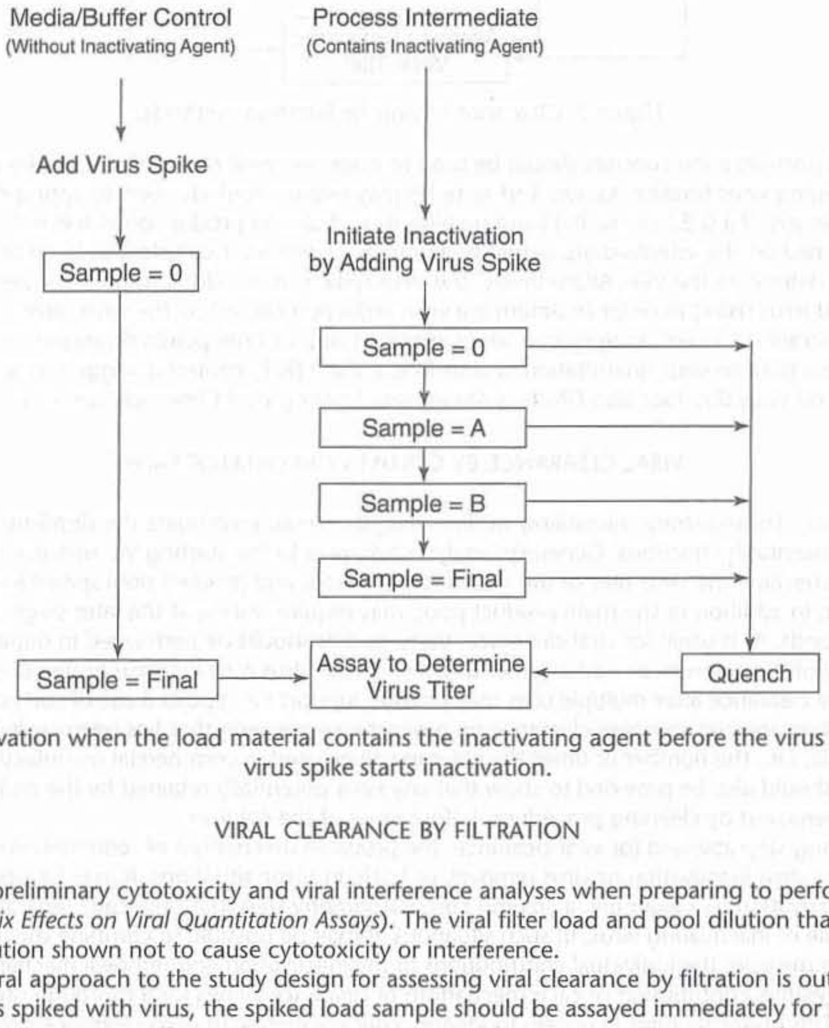


Figure 1B. Virus inactivation when the load material contains the inactivating agent before the virus spike; the addition of virus spike starts inactivation.

VIRAL CLEARANCE BY FILTRATION

Analysts must perform preliminary cytotoxicity and viral interference analyses when preparing to perform viral clearance by filtration (see *Sample Matrix Effects on Viral Quantitation Assays*). The viral filter load and pool dilution that will be used in the clearance study is that dilution shown not to cause cytotoxicity or interference.

Study design: The general approach to the study design for assessing viral clearance by filtration is outlined in *Figure 2*. Once the process intermediate is spiked with virus, the spiked load sample should be assayed immediately for virus titer. Virus spiking can be associated with aggregation effects. The aggregation status of a contaminating virus in the respective product intermediate is difficult to predict and therefore, as a worst-case approach, a mono-disperse virus preparation is advised for spiking. Filtration of virus before spiking (e.g., 0.45 µm or 0.22 µm) reduces larger virus aggregates from frozen virus stocks. A pure high-titer virus preparation can be helpful to reduce virus spike-induced effects influencing performance of virus filtration. The hold control sample should be maintained at processing conditions (temperature and time) and then assayed to evaluate the impact of these conditions, as well as the process intermediate components, on virus titer. Worst-case process conditions should be applied, if they have been established. The virus titers obtained from the spiked load and hold control should be within the experimental variability of the virus titration assay. If there is a negative impact on virus titer because of the processing conditions (temperature, time, and process intermediate composition), then an investigation should begin to understand the process. If warranted, the final VRF may need to be adjusted to account for the impact of hold conditions on viral prepara-

tions. However, this approach might underestimate the removal capacity from virus filtration. Alternatively, use of another detection method (e.g., NAT) or another, more stable, model virus can be discussed.

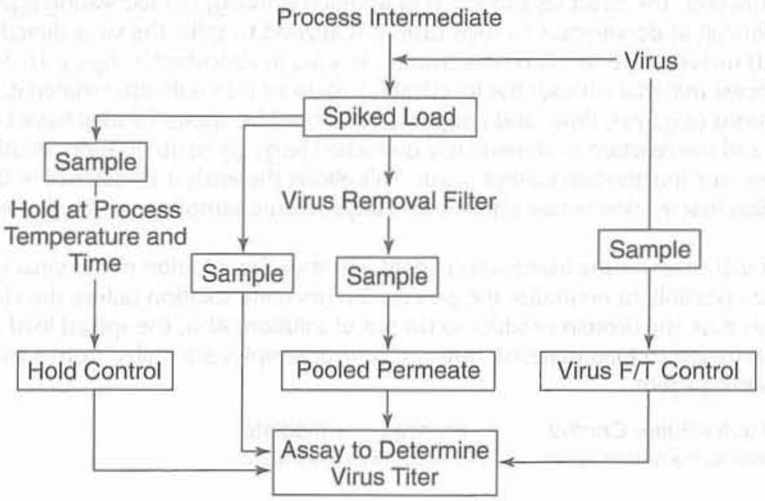


Figure 2. Clearance of virus by filtration methods.

Appropriate pre- and post-filtration controls should be used to assess removal of the virus spike by the prefiltration step. Samples are evaluated using virus titration assays. Test samples may require neutralization to appropriate pH levels for cell-culture-based titration assays. If a 0.22-µm or 0.45-µm prefilter is used during production in line with the virus-reduction filter, spiking could be performed on the intermediate before prefiltration. However, a sample should be taken from the stage after prefiltration in order to determine the VRF. Alternatively, the virus spike can be added to the intermediate after this prefiltration (using a similarly filtered virus spike) in order to determine virus reduction caused by the virus filter. To gain additional understanding of the virus clearance process, analysts can take samples at various time points throughout the run. In the event that samples are stored frozen prior to virus quantitation, a virus freeze/thaw (F/T) control is suggested to determine if there is any impact of the F/T cycle on virus titer (see also *Effects of Storing and Freezing Viral Clearance Samples*).

VIRAL CLEARANCE BY COLUMN CHROMATOGRAPHY

General considerations: For registration-enabling studies, analysts should investigate the distribution of the virus load among different chromatography fractions. Generally, analysts add virus to the starting intermediate (feed stream or load) for each step tested and determine the virus titer of the spiked load material and product pool spiked load material before and after the step. Fractions, in addition to the main product pool, may require testing at the later stages of product development (phase 3 trials and beyond). As is usual for viral clearance, these studies should be performed in duplicate. With column chromatography, the ability of the columns as well as other devices to clear virus may increase or decrease after repeated use. Evidence of consistent viral clearance after multiple uses may provide support for repeated use of such columns. Typically, this evidence is obtained when analysts compare clearance on new resin versus resin that has been cycled to or slightly beyond the targeted column lifetime, i.e., the number of times the column will be used in commercial manufacturing. For registration-enabling studies, data should also be provided to show that any virus potentially retained by the resin (carry-over) will be adequately inactivated or removed by cleaning procedures before reuse of the column.

For each manufacturing step assessed for viral clearance, the probable mechanism of reduction in viral infectivity should be known and described as virus inactivation or virus removal, or both. In some situations, it may be necessary to distinguish between removal and inactivation. For example, a column chromatography step that physically separates virus from product also may use a buffer capable of inactivating virus. In such situations, it may be possible to combine the use of viral infectivity assays with NAT assays to measure the individual contributions of the inactivation and removal mechanisms. Dissection of each step to determine the relative contribution of each mechanism of clearance allows for a thorough understanding of how viral clearance is achieved. This understanding may help to identify critical variables in each clearance step that should be controlled to support reproducible clearance. The testing of each critical variable (when it is well understood) at worst-case conditions helps to evaluate the process robustness of the step.

To measure virus clearance by a column chromatography system, analysts must perform preliminary cytotoxicity and viral interference analyses (see *Sample Matrix Effects on Viral Quantitation Assays* for details). The column load dilution that will be used in the clearance study is that dilution shown not to cause cytotoxicity or interference. [Note—Interference and cytotoxicity studies also can be performed on collected fractions, if warranted.]

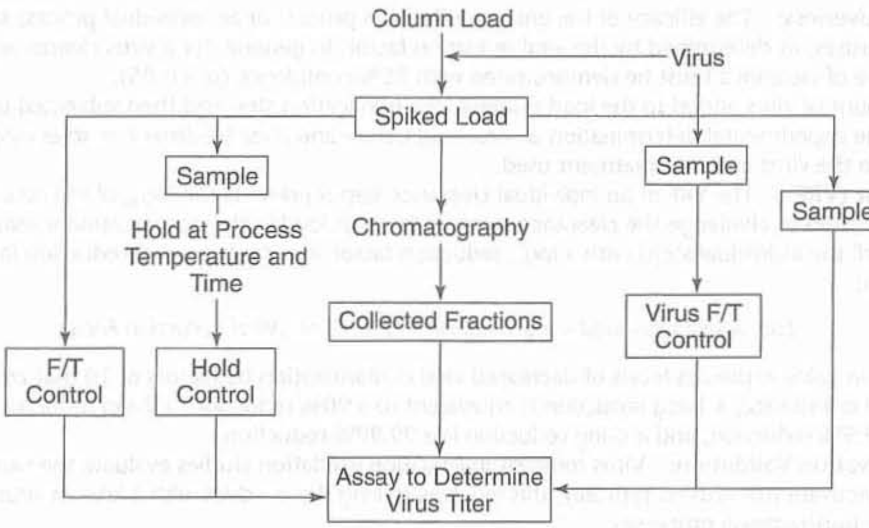


Figure 3. Clearance of virus by a column chromatography system.

Study design: *Figure 3* shows the general approach to study design for assessing viral clearance by chromatography. The column load is the material applied to the chromatography column. The load material in small-scale viral clearance studies should be comparable to material from clinical or commercial manufacturing in terms of composition and physicochemical attributes. The virus stock is a freshly thawed virus material of predetermined infectivity titer that is used to perform virus spiking of the column load material and also is used in the interference study. The virus stock can be sampled to determine the actual virus titer immediately after thaw. The spiked load control and additional samples should be tested immediately or immediately frozen. The titer of the frozen sample and other samples will be determined at the end of the chromatography step run. In certain cases, filtration of the spiked load with a 0.22-µm or 0.45-µm prefilter may be necessary to remove any viral aggregates formed when the process intermediate was spiked with the model virus.

The chromatography step experiment for viral clearance should closely mimic the actual manufacturing process step parameters. It should be performed under worst-case conditions (e.g., flow, protein concentration, and elution conditions) if they have been established. The product pool or multiple fractions can be collected, and the virus titer can be determined in each collected fraction (such as flow-through, washes, pre-peak, peak, post-peak, strip, and others). The titers of virus in all the fractions that comprise the product pool are used to calculate the clearance factor of the step. Interference and cytotoxicity studies also can be performed on collected fractions, if warranted. For the duration of the step, the hold control (the load plus virus) should be kept at the temperature at which the chromatography is performed. At the end of the chromatography run, the hold control should be titrated along with other collected samples/fractions (these can be titrated immediately, or if not feasible, can be frozen and tested at a later time). Samples are evaluated using virus titration assays. Test samples may require neutralization to appropriate pH levels for cell-culture-based titration assays.

GLOSSARY

(See also *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* ⟨1050⟩, *Glossary*.)[1]

Process Robustness of Viral Clearance: Ability of the downstream process or a single-unit operation to exhibit the characteristic performance and effectiveness in clearing viruses after minor or moderate changes of standard operating parameters or conditions.

Process Solutions: Production intermediates that are obtained for viral clearance studies. They should be obtained from a full-scale production facility or from a scaled-down process that has been shown to be representative of full-scale production.

Production Process: The manufacturing processes for biotechnology products are often varied and complex, and they are typically divided into upstream and downstream processing. Upstream activities produce the protein of interest, usually by cell culture or fermentation, and considerations include integrity and quality of the process, cell banks, expression systems, cultivation, media, process/product purity, impurities, and contaminants. Downstream processing involves separation and purification of the bulk bioproduct to make it suitable for its end use, e.g., purification, sterilization, and final formulation. Downstream activities include filtration, centrifugation, precipitation, numerous chromatographic separations, sterilization by terminal filtration, or lyophilization.

Purification Process: Separating and isolating the product of interest, in its desired form, from the fermentation supernatant or cell homogenate.

[1] Cf. glossary in ⟨1050⟩.

General Chapters

Viral Clearance Effectiveness: The efficacy of the entire purification process or an individual process step within a purification process to clear viruses, as determined by the viral reduction factor. In general, for a virus clearance step to be considered effective 4 logs or more of clearance must be demonstrated with 95% confidence ($\alpha = 0.05$).
Viral Load: The amount of virus added to the load material of a purification step and then subjected to an inactivation/removal treatment. The experimental determination of viral load before and after treatment enables calculation of a reduction factor that is specific to the virus and the treatment used.
Viral Reduction Factor (VRF): The VRF of an individual clearance step represents the $\log_{10}$ of the ratio of the virus load in the pretreatment material (used to challenge the clearance step) to the virus load in the post-treatment material. The overall VRF is calculated by adding all the individual steps with a $\log_{10}$ reduction factor >1. The $\log_{10}$ viral reduction factor is determined by the following equation:

$$Log_{10}initial\ virus\ load - log_{10}final\ virus\ load = Log_{10}Viral\ Reduction\ Factor$$

The $\log_{10}$ viral reduction value expresses levels of decreased viral contamination by factors of 10 that could be easily converted to percent reduction. For instance, a 1-log reduction is equivalent to a 90% reduction, a 2-log reduction is a 99% reduction, a 3-log reduction is a 99.9% reduction, and a 4-log reduction is a 99.99% reduction.
Virus Removal/Inactivation Validation: Virus removal/inactivation validation studies evaluate the capacity of the process to eliminate and/or to inactivate the viruses. Typically, this involves spiking the product with a known virus and then subjecting the product to inactivation/removal processes.

⟨1051⟩ CLEANING GLASS APPARATUS

Success in conducting many Pharmacopeial assays and tests depends upon the cleanliness of the glassware apparatus used. Usage of commercial detergents or inorganic reagents for cleaning should be used when necessary.

In all cases, it is important to verify that the cleaning procedure is appropriate for the particular test or assay being undertaken. This can be accomplished in a number of ways, including use of experimental controls or verification of cleaning by utilization of residue/residual testing to ensure removal of any potential contaminants. A statement should be included in the cleaning protocol describing how the success of the cleaning procedure will be assessed.

For optical measurements, special care is required for cleaning containers, but the use of chromic acid or highly alkaline solutions should be avoided.

Some particular tests, though not inclusive, wherein the use of clean glassware is critical for success include the following: pyrogen and total organic carbon tests as well as assays of heparin sodium and vitamin B_{12} activity.

Selected references that might be helpful in obtaining additional information on cleaning glass apparatus are listed in the *Appendix*. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about the cleanliness of the glassware apparatus procedures mentioned in this chapter may also be found in most quantitative chemical analytical textbooks.

APPENDIX

Additional Information

Additional information and guidance can be found in the references listed below or in many quantitative chemical analytical textbooks:

1. Parenteral Drug Association. *Draft—Points to Consider for Cleaning Validation* (Technical Report Number 29). Bethesda, MD: Parenteral Drug Association; 1998.
2. Anderson NR. Container cleaning and sterilization. In: Olson WP, Groves MJ, eds. *Aseptic Pharmaceutical Manufacturing.* 1st ed. Buffalo Grove, IL: Interpharm Press; 1987:15–22.
3. Green C. Cleaning validation—application in the laboratory; Montalvo M. The cleaning validation policy and the cleaning validation plan; Verghese G, Kaiser N. Cleaning agents and cleaning chemistry; Verghese G, Lopolito P. Cleaning engineering and equipment design. In: Pluta PL, ed. *Cleaning and Cleaning Validation, Volume 1*. Bethesda, MD: Parenteral Drug Association; 2009.
4. Gordon AJ, Ford RA. Standard glassware cleaning solutions. In: Gordon AJ, Ford RA, eds. *The Chemist's Companion*. Hoboken, NJ: Wiley and Sons; 1973.

⟨1052⟩ BIOTECHNOLOGY-DERIVED ARTICLES—AMINO ACID ANALYSIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by amino acid analysis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. The footnote below is in the *USP* but is not in the *EP* or *JP*. Other characterization tests, also harmonized, are shown in *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available* for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test

* ♦Suitable standards may be obtained from NIST (Gaithersburg, MD), Beckman Instruments (Fullerton, CA), Sigma Chemical (St. Louis, MO), Pierce (Rockford, IL), or Agilent (Palo Alto, CA).♦

General Chapters

material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and is used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often, the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to the quality of reagents and/or to laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reverse-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids from the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this particular point into consideration when interpreting the results. Internal standards can

also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 N hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC-grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500° for 4 hours may be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine, the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., *Methods 4–11*) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but it requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of 1 minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. [NOTE—During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.]

Method 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol.

Procedure—

Liquid Phase Hydrolysis—Place the protein or peptide sample in a hydrolysis tube, and dry. [NOTE—The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200 µL of *Hydrolysis Solution* per 500 µg of lyophilized protein. Freeze the sample tube in a dry ice–acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110° for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis—This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution*. The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum

(less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution: 2.5 M MESA solution.

Vapor Phase Hydrolysis—About 1 to 100 µg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution.* The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution.* The hydrolysis tube is heated to between 170° to 185° for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution: a solution containing 7 M hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis—About 10 to 50 µg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution.* The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166° for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

Method 4

Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation Solution—The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubating at room temperature for 1 hour.

Procedure—The protein/peptide sample is dissolved in 20 µL of formic acid, and heated at 50° for 5 minutes; then 100 µL of the *Oxidation Solution* is added. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The oxidation is allowed to proceed for 10 to 30 minutes. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2.*

Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis—The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis Solution.* This technique allows better tyrosine recovery than *Method 4,* but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis—The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution.* As an approach to limit variability and to compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

Method 7

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

Reducing Solution—Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container, and mix.

Procedure—Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 µm of mercury or 6.7 Pa), and incubate at about 100° for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine to improve accuracy in the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

Method 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions—Prepare and filter three solutions: 1 M Tris hydrochloride (pH 8.5) containing 4 mM edetate disodium (*Stock Solution 1*), 8 M guanidine hydrochloride (*Stock Solution 2*), and 10% of 2-mercaptoethanol in water (*Stock Solution 3*).

Reducing Solution—Prepare a mixture of *Stock Solution 2* and *Stock Solution 1* (3:1) to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M Tris hydrochloride.

Procedure—Dissolve about 10 µg of the test sample in 50 µL of the *Reducing Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

Method 9

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock Solutions—Prepare as directed for *Method 8*.

Carboxymethylation Solution—Prepare a solution containing 100 mg of iodoacetamide per mL of alcohol.

Buffer Solution—Use the *Reducing Solution*, prepared as directed for *Method 8*.

Procedure—Dissolve the test sample in 50 µL of the *Buffer Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the *Carboxymethylation Solution* in a 1.5 fold ratio per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [NOTE—If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethylcysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [NOTE—The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution: a solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 M sodium hydroxide.

Procedure—Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the *Reducing Solution*. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

Method 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by Asx, while glutamine and glutamic acid residues are added and represented by Glx. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions—Prepare and filter three solutions: a solution of 10 mM trifluoroacetic acid (*Solution 1*), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (*Solution 2*), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per mL (*Solution 3*).

Procedure—In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of *Solution 1* or *Solution 2* and 2 mL of *Solution 3*. Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α-, β-diaminopropionic and α-, γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid assayed contents with underivatized and BTI-derivatized acid hydrolysis. [NOTE—The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues.]

METHODOLOGIES OF AMINO ACID ANALYSIS GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenyl-methylchloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reverse-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, and chromatographic systems. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

Method 1—Postcolumn Ninhydrin Detection General Principle

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acids, give a purple color, and show maximum absorption at 570 nm. The imino acids, such as proline, give a yellow color, and show maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

Method 2—Postcolumn OPA Fluorometric Detection General Principle

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method I*. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this method exist.

Although OPA does not react with secondary amines (imino acids, such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound, such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes of pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limit is considered to be a few tens of pmol level for most of the amino acid derivatives. Response linearity is obtained in the range of a few pmol level to a few tens of nmol level. To obtain good compositional data, a sample greater than 500 ng before hydrolysis is best suited for the amino acid analysis of protein/peptide.

Method 3—Precolumn PITC Derivatization General Principle

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reverse-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, a sample larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino analysis of proteins/peptides.

Method 4—Precolumn AQC Derivatization General Principle

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reverse-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reverse-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reverse-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in the concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent byproduct, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ($t_{1/2}$< 15 seconds) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to be ranging from about 40 fmol to 320 fmol for each amino acid, except for Cys. The detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 µM to 200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

Method 5—Precolumn OPA Derivatization General Principle

Precolumn derivatization of amino acids with *o*-phthalaldehyde (OPA) followed by reverse-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol and 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reactions, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

Precolumn derivatization of amino acids with OPA is followed by reverse-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of the OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

General Chapters

The detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

Method 6—Precolumn DABS-Cl Derivatization General Principle

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reverse-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acid derivatives, can be separated on an ODS column of a reverse-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyze the imino acids, such as proline, together with the amino acids, at the same degree of sensitivity. DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids, such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, or methanesulfonic acid, described for *Method 2* in *Protein Hydrolysis*. The other acid-labile residues, asparagine and glutamine, can also be analyzed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI, described for *Method 11* in *Protein Hydrolysis*.

The nonproteinogenic amino acid, norleucine, cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analyzed with reliability, and only 10 ng to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

Method 7—Precolumn FMOC-Cl Derivatization General Principle

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reverse-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions, in aqueous solution, and is completed in 30 seconds. The derivatives are stable, with only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by reverse-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetic acid buffer, methanol, and acetonitrile (50:40:10) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives that are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 µM to 50 µM is obtained for most amino acids.

Method 8—Precolumn NBD-F Derivatization General Principle

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reverse-phase HPLC separation with fluorometric detection is used.

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reverse-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives that are separated in 35 minutes. *E*-aminocaproic acid can be used as an internal standard because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as that for the precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive and might be advantageous for NBD-F against OPA.

The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 mg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine

residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Calculations

Amino Acid Mole Percent—This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein/peptide under investigation is unknown. This information can be used to corroborate the identity of a protein and has other applications. Carefully identify and integrate the peaks obtained as directed for each *Procedure.* Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100r_U/r$$

in which r_U is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples—This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in µg, of each recovered amino acid by the formula:

$$mM_W/1000$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and M_W is the molecular weight, for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m/(1000M/M_{WT})$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in µg, of the protein; and M_{WT} is the molecular weight of the unknown protein.

Known Protein Samples—This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine), and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically, a greater than 5% variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m/m_s$$

in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and m_s is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

General Chapters

•APPENDIX

AMINO ACID ANALYSIS PROCEDURES

The examples of the specific procedures for each *Method* described in *Methodologies of Amino Acid Analysis* are shown.

Method 1—Postcolumn Ninhydrin Detection

One method for postcolumn ninhydrin detection is shown below. Many other methods are also available, with instruments and reagents available commercially.

Mobile Phase Preparation—

Solution A—Transfer about 1.7 g of anhydrous sodium citrate and 1.5 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 3.0.

Solution B—Transfer about 1.7 g of anhydrous sodium citrate and 0.7 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 4.3.

Solution C—Prepare a solution containing 5% of sodium chloride, 1.9% of anhydrous sodium citrate, and 0.1% of phenol in water, and adjust to a pH of 6.

Column Regeneration Solution—Prepare a solution containing 0.8% of sodium hydroxide in water, and adjust to a pH of 13.

Mobile Phase—Use variable mixtures of *Solution A, Solution B,* and *Solution C* as directed for *Chromatographic System.*

Postcolumn Reagent—Transfer about 18 g of ninhydrin and 0.7 g of hydrindantin to 900 mL of a solution containing 76.7% of dimethyl sulfoxide, 0.7% of dihydrate lithium acetate, and 0.1% of acetic acid, and mix for at least 3 hours under inert gas, such as nitrogen. [NOTE—This reagent is stable for 30 days if kept between 2° and 8° under inert gas.]

Buffer Solution—Prepare a solution containing 2% of anhydrous sodium citrate, 1% of hydrochloric acid, 0.5% of thiodiglycol, and 0.1% of benzoic acid in water, and adjust to a pH of 2.

Chromatographic System—The liquid chromatograph is equipped with a detector with appropriate interference filters at 440, 570, or 690 nm and a 4.0-mm × 120-mm column that contains 7.5-µm sulfonated styrene-divinylbenzene copolymer packing. The flow rate is about 14 mL per hour. The system is programmed as follows. Initially equilibrate the column with *Solution A;* at 25 minutes, the composition of the *Mobile Phase* is changed to 100% *Solution B;* and at 37 minutes, the composition is changed to 100% *Solution C.* At 75 minutes into the run, the last amino acid has been eluted from the column, and the column is regenerated with *Column Regeneration Solution* for 1 minute. The column is then equilibrated with *Solution A* for 11 minutes before the next injection. The column temperature is programmed as follows. The initial temperature is 48°; after 11.5 minutes, the temperature is increased to 65° at a rate of 3° per minute; at about 35 minutes, the temperature is increased to 77° at a rate of 3° per minute; and finally at about 52 minutes, the temperature is decreased to 48° at a rate of 3° per minute.

Procedure and Postcolumn Reaction—Reconstitute the lyophilized protein/peptide hydrolysate in the *Buffer Solution,* inject an appropriate amount into the chromatograph, and proceed as directed for *Chromatographic System.* As the amino acids are eluted from the column, they are mixed with the *Postcolumn Reagent,* which is delivered at a flow rate of 7 mL per hour, through a tee. After mixing, the column effluent and the *Postcolumn Reagent* pass through a tubular reactor at a temperature of 135°, where a characteristic purple or yellow color is developed. From the reactor, the liquid passes through a colorimeter with a 12-mm flow-through cuvette. The light emerging from the cuvette is split into three beams for analysis by the detector with interference filters at 440, 570, or 690 nm. The 690-nm signal may be electronically subtracted from the other signals for improved signal-to-noise ratios. The 440-nm (imino acids) and the 570-nm (amino acids) signals may be added in order to simplify data handling.

Method 2—Postcolumn OPA Fluorometric Detection

One method of postcolumn OPA fluorometric detection is shown below.

Mobile Phase Preparation—

Solution A—Prepare a solution of sodium hydroxide, citric acid, and alcohol in HPLC-grade water having a 0.2 N sodium concentration and containing 7% of alcohol (w/v), adjusted to a pH of 3.2.

Solution B—Prepare a solution of sodium hydroxide and citric acid in HPLC-grade water having a 0.6 N sodium concentration, adjusted to a pH of 10.0.

Solution C: 0.2 N sodium hydroxide.

Mobile Phase—Use variable mixtures of *Solution A, Solution B,* and *Solution C* as directed for *Chromatographic System.*

Postcolumn Reagent Preparation—

Alkaline Buffer—Prepare a solution containing 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, and adjust to a pH of 10.0.

Hypochlorite Reagent—To 1 L of *Alkaline Buffer,* add 0.4 mL of sodium hypochlorite solution (10% chlorine concentration). [NOTE—The hypochlorite solution is stable for 2 weeks.]

OPA Reagent—Transfer 2 g of *N*-acetyl-L-cysteine and 1.6 g of OPA to a 15-mL volumetric flask, dissolve in and dilute with alcohol to volume, and mix. Transfer this solution and 4 mL of 10% aqueous polyethylene (23) lauryl ether to a 1-L volumetric flask, dilute with 980 mL of *Alkaline Buffer,* and mix.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set to an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.0-mm × 150-mm column that contains 7.5-µm packing L17. The flow rate is about 0.3 mL per minute, and the column temperature is set at 50°. The system is programmed as follows. The column is equilibrated with *Solution A;* over the next 20 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B;* then there is a step change to 40% *Solution A* and 60% *Solution B;* over the next 18 minutes, the composition is changed linearly to 100% *Solution B* and held for 7 minutes; then there is a step change to 100% *Solution C,* and this is held for 6 minutes; then there is a step change to *Solution A,* and this composition is maintained for the next 8 minutes.

Procedure and Postcolumn Reaction—Inject about 1.0 nmol of each amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.* As the effluent leaves the column, it is mixed with the *Hypochlorite Reagent.* The mixture passes through the first postcolumn reactor which consists of stainless steel 0.5-mm × 2-m tubing. A second postcolumn reactor of similar design is placed immediately downstream from the first postcolumn reactor and is used for the OPA postcolumn reaction. The flow rates for both the *Hypochlorite Reagent* and the *OPA Reagent* are 0.2 mL per minute, resulting in a total flow rate (i.e., *Hypochlorite Reagent, OPA Reagent,* and column effluent) of 0.7 mL per minute exiting from the postcolumn reactors. Postcolumn reactions are conducted at 55°. This results in a residence time of about 33 seconds in the OPA postcolumn reactor. After postcolumn derivatization, the column effluent passes through the fluorometric detector.

Method 3—Precolumn PITC Derivatization

One method of precolumn PITC derivatization is described below.

Mobile Phase Preparation—

Solution A: 0.05 M ammonium acetate, adjusted with phosphoric acid to a pH of 6.8.

Solution B—Prepare 0.1 M ammonium acetate, adjust with phosphoric acid to a pH of 6.8, and then prepare a mixture of this solution and acetonitrile (1:1).

Solution C: a mixture of acetonitrile and water (70:30).

Mobile Phase—Use variable mixtures of *Solution A, Solution B,* and *Solution C* as directed for *Chromatographic System.*

Derivatization Reagent Preparation—

Coupling Buffer: a mixture of acetonitrile, pyridine, triethylamine, and water (10:5:2:3).

Sample Solvent: a mixture of water and acetonitrile (7:2).

Sample Derivatization Procedure—Dissolve the lyophilized test sample in 100 µL of the *Coupling Buffer,* and then dry in a vacuum centrifuge to remove any hydrochloride if a protein hydrolysis step was used. Dissolve the test sample in 100 µL of *Coupling Buffer,* add 5 µL of PITC, and incubate at room temperature for 5 minutes. The test sample is again dried in a vacuum centrifuge, and is dissolved in 250 µL of *Sample Solvent.*

Chromatographic System—The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 250-mm column that contains 5-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 52°. The system is programmed as follows. The column is equilibrated with *Solution A;* over the next 15 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B;* over the next 15 minutes, the composition is changed linearly to 50% *Solution A* and 50% *Solution B;* then there is a step change to 100% *Solution C,* and this is held for 10 minutes; then there is a step change to 100% *Solution A,* and the column is allowed to equilibrate before the next injection.

Procedure—Inject about 1.0 nmol of each PITC-amino acid under test (10-µL sample in *Sample Solvent*) into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 4—Precolumn AQC Derivatization

One method of precolumn AQC derivatization is shown below.

Mobile Phase Preparation—

Solution A—Prepare a solution having a composition of 140 mM sodium acetate and 17 mM triethylamine, and adjust with phosphoric acid to a pH of 5.02.

Solution B: a mixture of acetonitrile and water (60:40).

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System.*

Sample Derivatization Procedure—Dissolve about 2 µg of the test sample in 20 µL of 15 mM hydrochloric acid, and dilute with 0.2 M borate buffer (pH 8.8) to 80 µL. The derivatization is initiated by the addition of 20 µL of 10 mM AQC in acetonitrile, and allowed to proceed for 10 minutes at room temperature.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm and a 3.9-mm × 150-mm column that contains 4-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with *Solution A;* over the next 0.5 minute, the composition of the *Mobile Phase* is changed linearly to 98% *Solution A* and 2% *Solution B;* then over the next 14.5 minutes to 93% *Solution A* and 7% *Solution B;* then over

the next 4 minutes to 87% *Solution A* and 13% *Solution B;* over the next 14 minutes to 68% *Solution A* and 32% *Solution B;* then there is a step change to 100% *Solution B* for a 5-minute wash; over the next 10 minutes, there is a step change to 100% *Solution A;* and the column is allowed to equilibrate before the next injection.

Procedure—Inject about 0.05 nmol of each AQC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 5—Precolumn OPA Derivatization

One method of precolumn OPA derivatization is shown below.

Mobile Phase Preparation—

Solution A: a mixture of 100 mM sodium acetate (pH 7.2), methanol, and tetrahydrofuran (900:95:5).

Solution B: methanol.

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System.*

Derivatization Reagent—Dissolve 50 mg of OPA in 1.25 mL of methanol (protein sequencing grade). Add 50 µL of 2-mercaptoethanol and 11.2 mL of 0.4 M sodium borate (pH 9.5), and mix. [NOTE—This reagent is stable for 1 week.]

Sample Derivatization Procedure—Transfer about 5 µL of the test sample to an appropriate container, add 5 µL of the *Derivatization Reagent,* and mix. After 1 minute, add not less than 20 µL of 0.1 M sodium acetate (pH 7.0). Use 20 µL of this solution for analysis. [NOTE—Use of an internal standard (e.g., norleucine) is recommended for quantitative analysis because of potential reagent volume variations in the sample derivatization. The sample derivatization is performed in an automated online fashion. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization.]

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.6-mm × 75-mm column that contains 3-µm packing L3. The flow rate is about 1.7 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with 92% *Solution A* and 8% *Solution B;* over the next 2 minutes, the composition of the *Mobile Phase* is changed to 83% *Solution A* and 17% *Solution B,* and held for an additional 3 minutes; then changed to 54% *Solution A* and 46% *Solution B* over the next 5 minutes, and held for an additional 2 minutes; then changed to 34% *Solution A* and 66% *Solution B* over the next 2 minutes, and held for 1 minute; then over the next 0.3 minute changed to 20% *Solution A* and 80% *Solution B,* and held for an additional 2.6 minutes; and then finally over 0.6 minute changed to 92% *Solution A* and 8% *Solution B,* and held for an additional 0.6 minute.

Procedure—Inject about 0.02 nmol of each OPA-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 6—Precolumn DABS-Cl Derivatization

One method for precolumn DABS-Cl derivatization is shown below.

Mobile Phase Preparation—

Solution A: 25 mM sodium acetate (pH 6.5) containing 4% of dimethylformamide.

Solution B: acetonitrile.

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System.*

Derivatization Reagent Preparation—

Sample Buffer: 50 mM sodium bicarbonate, adjusted to a pH of 8.1.

Derivatization Reagent—Dissolve 1.3 mg of DABS-Cl in 1 mL of acetonitrile. [NOTE—This reagent is prepared fresh shortly before the derivatization step.]

Sample Dilution Buffer—Prepare a mixture of 50 mM sodium phosphate (pH 7.0) and alcohol (1:1).

Sample Derivatization Procedure—Dissolve the test sample in 20 µL of *Sample Buffer,* add 40 µL of *Derivatization Reagent,* and mix. The sample container is sealed with a silicon-rubber stopper, and heated to 70° for 10 minutes. During the sample heating, the mixture will become completely soluble. After the derivatization, dilute the test sample with an appropriate quantity of the *Sample Dilution Buffer.*

Chromatographic System—The liquid chromatograph is equipped with a 436-nm detector and a 4.6-mm × 250-mm column that contains packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 85% *Solution A* and 15% *Solution B;* over the next 20 minutes, the composition of the *Mobile Phase* is changed to 60% *Solution A* and 40% *Solution B;* over the next 12 minutes, the composition is changed to 30% *Solution A* and 70% *Solution B,* and held for an additional 2 minutes.

Procedure—Inject about 0.05 nmol of the DABS-amino acids into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 7—Precolumn FMOC-Cl Derivatization

One method for precolumn FMOC-Cl derivatization is shown below.

Mobile Phase Preparation—

Acetic Acid Buffer—Transfer 3 mL of glacial acetic acid and 1 mL of triethylamine to a 1-L volumetric flask, and dilute with HPLC-grade water to volume. Adjust with sodium hydroxide to a pH of 4.20.

Solution A: a mixture of *Acetic Acid Buffer,* methanol, and acetonitrile (50:40:10).

Solution B: a mixture of acetonitrile and *Acetic Acid Buffer* (50:50).

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System.*

Derivatization Reagent Preparation—

Borate Buffer—Prepare a 1 M boric acid solution, and adjust with sodium hydroxide to a pH of 6.2.

FMOC-Cl Reagent—Dissolve 155 mg of 9-fluorenylmethyl chloroformate in 40 mL of acetone, and mix.

Sample Derivatization Procedure—To 0.4 mL of the test sample add 0.1 mL of *Borate Buffer* and 0.5 mL of *FMOC-Cl Reagent.* After about 40 seconds, extract the mixture with 2 mL of pentane, and then extract again with fresh pentane. The aqueous solution with amino acid derivatives is then ready for injection.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm and a 4.6-mm × 125-mm column that contains 3-µm packing L1. The flow rate is about 1.3 mL per minute. The system is programmed as follows. The column is equilibrated with *Solution A,* and this composition is maintained for 3 minutes; over the next 9 minutes, it is changed to 100% *Solution B;* then over the next 0.5 minute, the flow rate is increased to 2 mL per minute, and held until the final FMOC-amino acid is eluted from the column. The total run time is about 20 minutes.

Procedure—Inject not less than 0.01 nmol of each FMOC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.* The FMOC-histidine derivative will generally give a lower response than the other derivatives.

Method 8—Precolumn NBD-F Derivatization

One method for precolumn NBD-F derivatization is shown below.

Mobile Phase Preparation—

Solution A: a solution of 10 mM sodium citrate containing 75 mM sodium perchlorate, adjusted with hydrochloric acid to a pH of 6.2.

Solution B: a mixture of acetonitrile and water (50:50).

Derivatization Reagent Preparation—

Sample Buffer: a 0.1 M boric acid solution, adjusted with sodium hydroxide to a pH of 9.2.

Derivatization Reagent—Dissolve 5 mg of NBD-F in 1.0 mL of alcohol, and mix.

Sample Derivatization Procedure—Dissolve the test sample in 20 µL of *Sample buffer,* add 10 µL of *Derivatization Reagent,* and mix. The sample container is heated at 60° for 5 minutes. After the derivatization, dilute the test sample with 300 µL of *Solution A.*

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm and a 4.6-mm × 150-mm column that contains 5-µm particle size ODS silica packing. The flow rate is about 1.0 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 94% *Solution A* and 6% *Solution B;* over the next 16 minutes, the composition is changed linearly to 63% *Solution A* and 37% *Solution B;* over the next 5 minutes, the composition is changed linearly to 62% *Solution A* and 38% *Solution B;* over the next 9 minutes, the composition is changed linearly to 100% *Solution B,* and held for an additional 5 minutes; then finally over 2 minutes, the composition is changed linearly to 94% *Solution A* and 6% *Solution B;* and then the column is allowed to equilibrate before the next injection.

Procedure—Inject about 15 pmol of each NBD-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.*▲

〈1053〉 CAPILLARY ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by capillary electrophoresis. This chapter is harmonized with the corresponding chapter in *JP* and *EP.* Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* 〈1052〉, *Biotechnology-Derived Articles—Isoelectric Focusing* 〈1054〉, *Biotechnology-Derived Articles—Peptide Mapping* 〈1055〉, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* 〈1056〉, and *Biotechnology-Derived Articles—Total Protein Assay* 〈1057〉.

INTRODUCTION

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution under the influence of a direct-current electric field. In this section we are describing four capillary electrophoresis methods: *Capillary Zone Electrophoresis, Capillary Gel Electrophoresis, Capillary Isoelectric Focusing,* and *Micellar Electrokinetic Chromatography.*

GENERAL PRINCIPLES

The migration velocity of the analyte under an electric field of intensity E is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electrical charge, molecular size, and shape) and the characteristics of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity, and additives). The electrophoretic velocity (v_{ep}) of a solute, assuC159341_150319-M99797-GCPA2010heming a spherical shape, is as follows:

$$v_{ep} = \mu_{ep} E = \left(\frac{q}{6\pi\eta r}\right)\left(\frac{V}{L}\right)$$

in which q is the effective charge of the solute; η is the viscosity of the electrolyte solution; r is the Stoke's radius of the solute; V is the applied voltage; and L is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent, called electroosmotic flow, is generated inside the capillary. Its velocity depends on the electroosmotic mobility (μ_{eo}), which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} E = \left(\frac{\varepsilon\zeta}{\eta}\right)\left(\frac{V}{L}\right)$$

in which ε is the dielectric constant of the buffer; ζ is the zeta potential of the capillary surface; and the other terms are as defined above.

The velocity of the solute (v) is given by the equation:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electroosmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run. The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is as follows:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l(L)}{V(\mu_{ep} + \mu_{eo})}$$

in which the other terms are as defined above.

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electroosmotic flow is from anode to cathode. The electroosmotic flow must remain constant from run to run to obtain good reproducibility in the migration velocity of the solutes. For some applications, it might be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition, and/or the pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions, the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case, the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo})(Vl)}{2DL}$$

in which D is the molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unleveled buffer reservoirs, can also significantly contribute to band dispersion. Separation between two bands (expressed by the resolution, R_S) can be obtained by modifi-

cation of the electrophoretic mobility of the analytes, by the electroosmotic mobility induced in the capillary, and by increasing the efficiency for the band of each analyte as follows:

$$R_S = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\bar{\mu}_{ep} + \mu_{eo})}$$

in which μ_{epa} and μ_{epb} are the electrophoretic mobilities of the two analytes to be separated; $\bar{\mu}_{ep}$ is the average electrophoretic mobility of the two analytes calculated as:

$$\bar{\mu}_{ep} = \frac{1}{2}(\mu_{epb} + \mu_{epa})$$

APPARATUS

An apparatus for capillary electrophoresis is composed of a high voltage controllable direct current power supply; two buffer reservoirs held at the same level and containing specified anodic and cathodic solutions; two electrode assemblies (cathode and anode) immersed in the buffer reservoirs and connected to the power supply; a separation capillary usually made of fused-silica, sometimes with an optical viewing window aligned with the detector, depending on the detector type, with the ends of the capillary placed in the buffer reservoirs and the capillary being filled with a solution specified in a given monograph; a suitable injection system; a detector capable of monitoring the amount of substance of interest passing through a segment of the separation capillary at a given time, generally based on absorption spectrophotometry (UV and visible), fluorimetry, conductimetric, amperometric, or mass spectrometric detection, depending on the specific applications, or even indirect detection to detect non-UV-absorbing and nonfluorescent compounds; a thermostatic system capable of maintaining a constant temperature inside the capillary, recommended to obtain good separation reproducibility; a recorder; and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum, or electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

It is expected that the capillary, the buffer solutions, the preconditioning method, the sample solution, and the migration conditions will be specified in the individual monograph. The electrolytic solution employed is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. To achieve reproducible migration time of the solutes, it would be necessary to develop, for each analytical method, a rigorous rinsing routine.

CAPILLARY ZONE ELECTROPHORESIS

Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. In this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow on the capillary (see *General Principles*). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

This mode of capillary electrophoresis is appropriate for the analysis of small (MW < 2000) and large (2000 < MW < 100,000) molecules. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of the separations are instrumental and electrolytic solution parameters.

Instrumental Parameters

VOLTAGE

A Joule heating plot is useful in optimizing the applied voltage and column temperature. The separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to

temperature and, as a result, viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

POLARITY

Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

TEMPERATURE

The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

CAPILLARY

The length and internal diameter of the capillary affects the analysis time, the efficiency of separations, and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which increases migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic, and anionic polymers are commercially available.

Electrolytic Solution Parameters

BUFFER TYPE AND CONCENTRATIONS

Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

To minimize band distortion, it is important to match buffer–ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH decreases electroosmotic flow and solute velocity.

BUFFER pH

The pH of the buffer can affect separation by modifying the charge of the analyte or additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point to below the isoelectric point changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

ORGANIC SOLVENTS

Organic modifiers, such as methanol, acetonitrile, and others, may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

ADDITIVES FOR CHIRAL SEPARATIONS

To separate optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides, or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral

(methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutylether, etc.) moities. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account because it will influence the selectivity. The resolution of chiral separations is also controlled by the concentration of the chiral selector, the composition and pH of the buffer, and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution of separation.

CAPILLARY GEL ELECTROPHORESIS

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size because smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

Characteristics of Gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels are prepared inside the capillary by polymerization of monomers. One example of such a gel is a cross-linked polyacrylamide. This type of gel is usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. For protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate, and the sample is denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When nonreducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Optimization of separation in a cross-linked gel is obtained by modifying the separation buffer (see *Capillary Zone Electrophoresis*) and by controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the ratio of the cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of this type of gel, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers (i.e., linear polyacrylamide, cellulose derivatives, dextran, etc.) which can be dissolved in aqueous separation buffers, giving rise to a separation medium that also acts as a molecular sieve. These polymeric separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary with no electroosmotic flow. Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the dynamically coated gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A decrease in gel porosity leads to a decrease in the mobility of the solute for the same buffer. Both hydrodynamic and electrokinetic injection techniques can be used because the dissolution of these polymers in the buffer gives low viscosity solutions.

CAPILLARY ISOELECTRIC FOCUSING

Principle

In isoelectric focusing the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (polyaminocarboxylic acids), dissolved in the separation buffer.

The three basic steps in capillary isoelectric focusing are loading, focusing, and mobilization.

LOADING

Two methods may be employed.

Loading in One Step—The sample is mixed with ampholytes and introduced into the capillary by pressure or vacuum.

Sequential Loading—A leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone, and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough so as not to modify the pH gradient.

FOCUSING

When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charge, creating the pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point, and the current drops to very low values.

General Chapters

MOBILIZATION

If mobilization is required for detection, use one of the following methods. Three methods are available.

Method 1—Mobilization is accomplished during *Focusing*, under the influence of the electroosmotic flow when this flow is small enough to allow the focusing of the components.

Method 2—Mobilization is accomplished by application of positive pressure after *Focusing*.

Method 3—Mobilization is achieved after *Focusing*, by adding salts to the cathode reservoir or the anode reservoir, depending on the direction chosen for mobilization, in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir, which contains added salts, and pass the detector.

The separation achieved is expressed as ΔpI and depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E), and the variation of the electrophoretic mobility of the analyte with the pH (−dμ/dpH):

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

Optimization

The major parameters that need to be considered in the development of separations are the following:

VOLTAGE

The use of high fields from 300 V/cm to 1,000 V/cm during *Focusing*.

CAPILLARY

Depending on the *Mobilization* strategy selected (see above), the electroosmotic flow must be reduced or suppressed. Coated capillaries tend to reduce the electroosmotic flow.

SOLUTIONS

The anode buffer reservoir is filled with a solution of a lower pH than the pI of the most acidic ampholyte, and the cathode reservoir is filled with a solution with a higher pH than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, like methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes covering many pH ranges are available and may also be mixed to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point (pI), whereas narrower ranges are employed to improve accuracy. Calibration can be made by correlating migration time with the isoelectric point of a series of standard protein markers. During *Focusing*, precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea, or zwitterionic buffers. However, depending on the concentration, urea can denature proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

Principle

Separation takes place in an electrolytic solution that contains a surfactant at a concentration above the critical micellar concentration (cmc). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed by the micelles according to the solute's partition coefficient. The technique can be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes maintaining the efficiency, speed, and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant, sodium dodecyl sulfate, although other surfactants, such as cationic surfactant cetyl trimethyl ammonium salts, have also been used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is used as surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, because the analyte can partition between the micelle and the aqueous buffer and has no electrophoretic mobility, the analyte migration velocity will de-

pend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electroosmotic flow marker and that of the micelle; and the time elapsed between these two peaks is called the separation window. For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer and on the electrophoretic mobility of the solute in the absence of micelles.

Because the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k′), also referred to as mass distribution ratio (D_m), which is the ratio between the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k′ is as follows:

$$k' = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} = K\left(\frac{V_S}{V_M}\right)$$

in which t_r is the migration time of the solute; t_0 is the analysis time of the unretained solute obtained by injecting an electroosmotic flow marker that does not enter the micelle (e.g., methanol); t_{mc} is the micelle migration time measured by injecting a micelle marker, such as Sudan III, which migrates continuously associated in the micelle; K is the partition coefficient of the solute; V_S is the volume of the micellar phase; and V_M is the volume of the mobile phase.

The resolution between two closely-migrating solutes (R_S) is as follows:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b'}{k_b' + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k_a' \times \left(\frac{t_0}{t_{mc}}\right)}$$

in which N is the number of theoretical plates for one of the solutes; α is the selectivity; and k_a' and k_b' are retention factors for both solutes, respectively ($k_b' > k_a'$).

Similar, but not identical, equations give k′ and R_S values for electrically charged solutes.

Optimization

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

INSTRUMENTAL PARAMETERS

Voltage—Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross section of the capillary. This effect can be significant with high conductivity buffers, such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature—Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration, and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary—As in *Capillary Zone Electrophoresis,* length and internal diameter of the capillary contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electrical fields, working at constant voltage, and will increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation, for a given buffer and electrical field, and consequently broadening of the sample band.

ELECTROLYTIC SOLUTION PARAMETERS

Surfactant Type and Concentration—The type of surfactant, as the stationary phase in chromatography, affects the resolution because it modifies separation selectively. The log k′ of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. When k′ approaches the value of

$$\sqrt{t_{mc}/t_0}$$

resolution in MEKC reaches a maximum. Modifying the concentration of surfactant in the mobile phase changes the resolution.

Buffer pH—pH does not modify the partition coefficient of nonionized solutes, but it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and, therefore, increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic Solvents—To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers generally decreases migration time and

selectivity of the separation. The addition of organic modifiers affects critical micellar concentration, thus, a given surfactant concentration can be used only with a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, the absence of the partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible, because in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for Chiral Separations—For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts, *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions that contain micellized achiral surfactants.

Other Additives—Selectivity can be modified by adding chemicals to the buffer. Addition of several types of cyclodextrins to the buffer is also used to reduce the interaction of hydrophobic solutes with the micelle, increasing the selectivity for this type of compound. The addition of substances able to modify solute-micelle interactions by adsorption on the latter has been used to improve the selectivity of the separations in MEKC. These additives may consist of a second surfactant (ionic or nonionic), which gives rise to mixed micelles or metallic cations that dissolve in the micelle and form coordination complexes with the solutes.

Quantification

Peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response. Dividing the peak areas by migration time will also compensate for the different responses of sample constituents with different migration times. Where an internal standard is used, check that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of a component or components being determined. When indicated, the percentage of one (or more) components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all the peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. The parameters include the following: retention factor k′ used only for *Micellar Electrokinetic Chromatography,* apparent number of theoretical plates (N), the symmetry factor (A_S), and the resolution (R_S). Note that in previous sections, the theoretical expressions for N and R_S have been described, but more practical equations that allow for the determination of these suitability parameters using the electropherograms are described below.

Apparent Number of Theoretical Plates

The apparent number of theoretical plates (N) may be calculated from the formula:

$$N = 5.54\ (t_R/w_h)^2$$

in which t_R is the migration time or distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak corresponding to the component; and w_h is the peak width at half-height.

Resolution

The resolution (R_S) between peaks of similar heights of two components may be calculated from the formula:

$$R_S = 1.18\ (t_{R2} - t_{R1})/(w_{h1} + w_{h2})$$

$$t_{R2} > t_{R1}$$

in which t_{R1} and t_{R2} are the migration times or distances along the baseline between the point of injection and the perpendiculars dropped from the maxima of two adjacent peaks; and w_{h1} and w_{h2} are the peak widths at half-height.

When appropriate, the resolution (R_s) may also be calculated by measuring the height of the valley (H_v) between two partly resolved peaks in a standard preparation, the height of the smaller peak (H_p), and calculating the peak-to-valley ratio:

$$p/v = H_p/H_v$$

Symmetry Factor

The symmetry factor of a peak (A_s) may be calculated using the formula:

$$A_s = w_{0.05}/2d$$

in which $w_{0.05}$ is the width of the peak at one-twentieth of the peak height; and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Other suitability parameters include tests for area repeatability (standard deviation of areas or of area/migration time) and tests for migration time repeatability (standard deviation of migration time). Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use a migration time relative to an internal standard.

Signal-to-Noise Ratio

A test for the verification of the signal-to-noise ratio for a standard preparation or the determination of the limit of quantification may also be useful for the determination of related substances. The detection limit and quantification limit correspond to a signal-to-noise ratio of 3 and 10, respectively. The signal-to-noise ratio (S/N) is calculated as follows:

$$S/N = 2H/h$$

in which H is the height of the peak corresponding to the component concerned in the electropherogram obtained with the specified reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height; and h is the range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

⟨1054⟩ BIOTECHNOLOGY-DERIVED ARTICLES—ISOELECTRIC FOCUSING

This chapter provides guidance and procedures used for the characterization of biotechnology-derived articles by isoelectric focusing. This chapter is harmonized with the corresponding chapters in *JP* and *EP*. Other characterization tests, also harmonized, are shown in the USP general information chapters *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Biotechnology-Derived Articles—Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric points. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electrical field, the ampholytes migrate in the gel to create a pH gradient. In some cases, gels containing an immobilized pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralized and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentration effect is called "focusing". Increasing the applied voltage or reducing the sample load results in improved separation of bands. The applied voltage is limited by the heat generated because the

heat must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel while allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate two neighboring bands, as follows:

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

in which D is the diffusion coefficient of the protein; dpH/dx is the pH gradient; E is the intensity of the electric field, in volts per centimeter; and −dμ/dpH is the variation of the solute mobility with the pH in the region close to the pI. Because D and −dμ/dpH for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Better resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pI values differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes, whereas immobilized pH gradients can resolve protein differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

From an operational point, special attention must be paid to sample characteristics and/or preparation. Salt in a sample can be problematic, and it is best to prepare the sample, if possible, in deionized water or 2% ampholytes using dialysis or gel filtration if necessary. The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a colored protein (e.g., hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some procedures the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins; the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

APPARATUS

An apparatus for isoelectric focusing consists of a controllable generator for constant potential, current, and power. Potentials of 2500 V have been used and are considered optimal under a given set of operating conditions. Supply of up to 30 W of constant power is recommended. The apparatus also includes a rigid plastic isoelectric focusing chamber that contains a cooled plate of suitable material to support the gel; and a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length, and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

Preparation of the Gels

MOLD

The mold is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D), and clamps to hold the structure together (see *Figure 1*).

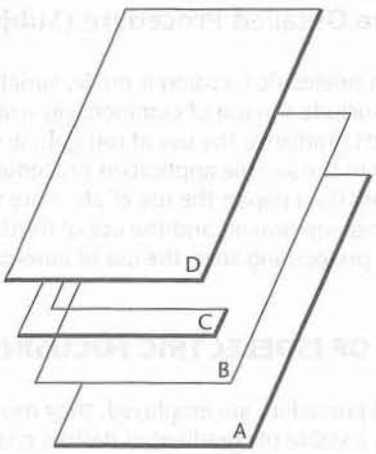


Figure 1. Mold

7.5% POLYACRYLAMIDE GEL

Dissolve 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the individual monograph, and dilute to 10 volumes with water. Mix carefully, and degas the solution.

PREPARATION OF THE MOLD

Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate, and fit the clamps. Before use, place the mixture on a magnetic stirrer, and add 0.25 volumes of a 100 g/L solution of ammonium persulfate and 0.25 volumes of tetramethylenediamine. Immediately fill the space between the glass plates of the mold with the solution.

FIXING SOLUTION FOR ISOELECTRIC FOCUSING POLYACRYLAMIDE GEL

Mix 35 g of sulfosalicylic acid and 100 g of trichloroacetic acid in 1000 mL of water.

COOMASSIE STAINING SOLUTION AND DESTAINING SOLUTION

Use the same solutions indicated in general information chapter *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* 〈1056〉.

Procedure

Dismantle the *Mold,* and using the polyester film, transfer the gel onto the cooled support wetted with a few mL of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the individual monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel, and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some procedures, the gel has precast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut two strips of paper to the length of the gel, and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the individual monograph. Apply these paper wicks to each side of the gel several mm from the edge. Fit the cover so that the electrodes are in contact with the wicks (with respect to the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the individual monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forceps, remove the sample application strips and the two electrode wicks. Immerse the gel in *Fixing Solution for Isoelectric Focusing Polyacrylamide Gel.* Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution, and add 200 mL of *Destaining Solution.* Incubate with shaking for 1 hour. Drain the gel, and add *Coomassie Staining Solution.* Incubate for 30 minutes. Destain the gel by passive diffusion with *Destaining Solution* until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram, as prescribed in the individual monograph.

Variations to the Detailed Procedure (Subject to Validation)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These variations include the use of commercially available precast gels and of commercial staining and destaining kits; the use of immobilized pH gradients; the use of rod gels, and the use of cassettes of different dimensions, including ultra-thin (0.2 mm) gels; variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper; the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability; the inclusion of a prefocusing step; the use of automated instrumentation; and the use of agarose gels.

VALIDATION OF ISOELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed, they must be validated. The following criteria may be used to validate the separation: formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points; comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined; and any other validation criteria as prescribed in the individual monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in individual monographs. Variations may include the addition of urea in the gel (a 3 M concentration is often satisfactory to keep the protein in solution, but up to 8 M can be used). Some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein. Other variations include the use of alternative staining methods and the use of gel additives such as nonionic detergents (e.g., octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO) and the addition of ampholyte to the sample to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

1. Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments, samples should not be applied close to either electrode. During method development, the analyst can try applying the protein in three positions on the gel (e.g., middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.
2. A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilized pH gradients may be used to address this problem.
3. Efficient cooling (approximately 4°) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

〈1055〉 BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by peptide mapping. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* 〈1052〉, *Capillary Electrophoresis* 〈1053〉, *Biotechnology-Derived Articles—Isoelectric Focusing* 〈1054〉, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* 〈1056〉, and *Biotechnology-Derived Articles—Total Protein Assay* 〈1057〉.

INTRODUCTION

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the formation of peptide fragments, followed by separation and identification of the resultant fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a Reference Standard or Reference Mate-

rial similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product or to detect the presence of protein variant. •The validation scheme presented differentiates between qualification of the method at an early stage in the regulatory process, the Investigational New Drug (IND) level, and full validation in support of New Drug Application (NDA), Product License Application (PLA), or Marketing Authorization Application (MAA). The validation concepts described are consistent with the general information chapter *Validation of Compendial Procedures* 〈1225〉 and with the International Conference on Harmonization (ICH) document *Analytical Methods Validation*.•

THE PEPTIDE MAP

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a Reference Standard or Reference Material. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in *Table 1*. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	C-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin, EC 3.4.23.1 and EC 3.4.23.2	Nonspecific digest
	Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50	C-terminal side of Lys
	Glutamyl endopeptidase; (from *S. aureus* strain V8), EC 3.4.21.19	C-terminal side of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Asp-N endoproteinase), EC 3.4.24.33	*N*-terminal side of Asp
	Clostripain, EC 3.4.22.8	C-terminal side of Arg
Chemical	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thiocyanobenzoic acid	*N*-terminal side of Cys
	o-Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

General Chapters

Pretreatment of Sample

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the Cleavage Agent

Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the Protein

Under certain conditions, it might be necessary to concentrate the sample, or to separate the protein from added substances and stabilizers used in the formulation of the product if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map as a result of side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of Optimal Digestion Conditions

Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH—The digestion mixture pH is empirically determined to ensure the optimal performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

Temperature—A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4° because at higher temperatures it will precipitate during digestion.

Time—If a sufficient amount of sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid that does not interfere with the tryptic map, or by freezing.

Amount of Cleavage Agent—Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein-to-protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents except the test protein.

Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for the separation of peptides are shown in *Table 2*.

Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)

General Chapters

Table 2. Techniques Used for the Separation of Peptides *(Continued)*

Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography—High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

In this section, a most widely used reverse-phase HPLC (RP-HPLC) method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration are also recommended.

Chromatographic Column—The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size and silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica particles 3 to 10 µm in diameter (L7) and of octadecylsilane chemically bonded to porous silica or ceramic microparticles 3 to 10 µm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles 5 to 10 µm in diameter (L26) packing.

Solvent—The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% of trifluoroacetic acid is added. If necessary, add isopropyl alcohol or *n*-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile Phase—Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, because shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile-containing trifluoroacetic acid is also used quite often.

Gradient Selection—Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become "marker" peaks for the test.

Isocratic Selection—Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

Other Parameters—Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or as versatile as UV detection.

System Suitability—The section *System Suitability* under *Chromatography* 〈621〉 provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is the comparison with a Reference Standard or Reference Material, which is treated exactly as the article under test. The use of a USP Reference Standard in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram should be included with the USP Reference Standard or Reference Material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection, and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant proteins is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the USP Reference Standard or Reference Material for the specified protein. The use of a digested USP Reference Standard or Reference Material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a Reference Standard can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the USP Reference Standard or Reference Material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and USP Reference Standard or Reference Material digest. If all peaks in the sample digest and in the USP Reference Standard or Reference Material digest have the same relative retention times and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and USP Reference Standard or Reference Material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches, for example the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides, have been proposed. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between USP Reference Standard or Reference Material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and is likely to introduce error into the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the USP Reference Standard or Reference Material. The possibility of autohydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when *N*-terminal sequencing and amino acid analysis are used, the analytical separation is scaled up. Because scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to ensure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase digestion and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF analyzers as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

•THE USE OF PEPTIDE MAPPING FOR GENETIC STABILITY EVALUATION

A validated peptide map can be used to assess the integrity of the predicted primary sequence of a protein product (i.e., its genetic stability). It can also be used to determine lot-to-lot consistency of the biotechnology-derived product process. Furthermore, the performance of the protein expression of the production system is best assessed by peptide mapping of the expressed protein. Peptide maps of protein produced at various times of the protein expression process, including a point well beyond the normal protein expression time, compared with those of a USP Reference Standard or Reference Material, will evaluate the genetic stability of the expression system as a function of time.

Variant protein sequences can arise from a genetic variation at the DNA level (point mutation) or as an error in the translation process. A validated peptide map is the best approach to the detection of protein variants. However, the limitations of the peptide mapping itself must be taken into consideration. The detection of a structured variant is possible only if the corresponding peptide variant is easily isolated and characterized. To establish genetic stability will require the use of a battery of biochemical methods, provided that the variants have properties different from those of the "normal" protein.•

•VALIDATION

Critical Factors

Validation of peptide mapping requires that a protocol be designed, outlining in detail the experiment to be conducted and the criteria for acceptance of the map. Criteria for acceptance of mapping include detection limit, specificity, linearity, range, accuracy, precision, and reagent stability. Reproducibility of the peptide map is a critical element in the utilization of such a map as an identity test and for confirming genetic stability. Those technical aspects of peptide mapping that influence the reproducibility of the map will be discussed.

The setting of limits, with respect to quantification (peak area or height) and identification (retention times) for the selected group of relevant peaks is based on empirical observations. These limits detect significant differences between the sample and USP Reference Standard or Reference Material within a series of analyses.

Another critical issue is the recovery of peptides and its impact on peak area determination and reproducibility and on the establishment of acceptance criteria. The recovery criteria address all aspects of test methodology, from digestion to chromatographic conditions. Determination of peptide recovery includes quantitative amino acid analysis, spike addition, radiolabeling, and UV summation. An overall recovery of about 80% is considered satisfactory. Recovery of individual peptides is more problematic and is handled on a case-by-case basis. The critical factors considered in the validation of a peptide map are as follows.

Written Test Procedures—These procedures include a detailed description of the analytical method in which reagents, equipment, sample preparation, method of analysis, and analysis of the data are defined.

Validation Protocol—A protocol is prepared that contains a procedure for test validation.

Acceptance Criteria—The criteria can be minimal at the early stages, but need to be better defined as validation studies progress.

Reporting of Results—Results from the validation study are documented with respect to the analytical parameters listed in the validation protocol.

Revalidation of the Test Procedure—If the method used requires alteration that could affect the analytical parameter previously assessed in the validation of the procedure, the test procedure must be revalidated. Significant changes in the processing of the article, in laboratories performing the analysis, in formulation of the bulk or the finished products, and in any other significant parameter will require revalidation of the methods.

Requirements

Precision

Intratest Precision—This is a measure of the reproducibility of peptide mapping. The two critical steps in peptide mapping are fragmentation (i.e., digestion) and separation of peptides. An acceptable precision occurs where the absolute retention times and the relative peak areas are constant from run to run, and the average variation in retention time is small relative to that of a selected internal reference peak. The reproducibility of the map can be enhanced if a temperature-controlled column oven is used, if an extensive equilibration of the system is performed prior to the start of the test, if a blank (control digest mixture without protein) is run first to minimize "first run effects," and if a USP Reference Standard or a Reference Material digest is interspersed periodically with test samples to evaluate chromatographic drift.

The criteria for validation of the fragmentation step are similar to those described below for separation of peptides, but they are met for consecutive tests of a series of separately prepared digests of the protein under test.

The criteria for validation of the separation of peptides step include the following:

1. The average standard deviation of the absolute retention times of all major peaks for a set of consecutive tests of the same digest does not exceed a specified acceptance criterion.

2. The average standard deviation of absolute peak area for all fully resolved major peaks does not exceed a specified percentage.

Intertest Precision—This is a measure of the reproducibility of the peptide mapping when the test is performed on different days, by different analysts, in different laboratories, with reagents or enzymes from different suppliers or different lots from the same supplier, with different instruments, on columns of different makes or columns of the same make from different lots, and on individual columns of the same make from the same lot. Although it would be desirable, from a scientific perspective, to validate all of these variables in terms of their impacts on precision, a practical approach is to validate the test using those variables most likely to be encountered under operational conditions. Additional variables can be included when needed.

The experimental design allows the analyst to make comparisons using peak retention times and areas that are expressed relative to a highly reproducible internal reference peak within the same chromatogram. The relative peak area is expressed as the ratio of the peak area to that of the internal reference peak. The relative retention time can be expressed as the difference between the absolute retention time and that of the reference peak. The use of relative values eliminates the need to make separate corrections for differences due to injector-to-injector volumes, units of measure for peak areas, column dimensions, and instrument dead volumes. The variability in the retention times and peak areas for the *Intertest Precision* experiments is expected to be slightly higher than the variability observed for *Intratest Precision*.

Robustness

Factors such as composition of the *Mobile Phase*, protease quality or chemical reagent purity, column variation and age, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

Mobile Phase—The composition of the *Mobile Phase* is optimized to obtain the maximum resolution of peptides throughout the elution profile. A balance between optimal resolution and overall reproducibility is desired. A lower pH might improve peak separation but might shorten the life of the column, resulting in lack of reproducibility. Peptide maps at a pH above and below the pH of the procedure are compared to the peptide map obtained at the pH of the procedure and checked for significant differences; they are also reviewed with respect to the acceptance criteria established in the validation protocol.

Protease Quality or Chemical Reagent Purity—A sample of the USP Reference Standard or Reference Material for the protein under test is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and carboxymethylation reagents.

Column Considerations—Column-to-column variability, even within a single lot, can affect the performance of the column in the development of peptide maps. Column size may also lead to significant differences. A USP Reference Standard or Reference Material of the protein under test is digested and the digest is chromatographed on different lots of column from a single manufacturer. The maps are then evaluated in terms of the overall elution profile, retention times, selectivity resolution, and recovery. To evaluate the overall lifetime of the column in terms of robustness, perform a peptide mapping test on different columns and vary significantly the number of injections (e.g., from 10 injections to 250 injections). The resulting maps can then be compared for significant differences in peak broadening, peak area, and overall resolution. As a column ages, an increase in back pressure might be observed that might affect the peptide maps.

A sensible precaution in the use of peptide mapping columns is to select alternative columns in case the original columns become unavailable or are discontinued. Perform a peptide mapping test using equivalent columns from different manufacturers, and examine the maps. Differences in particle shape and size, pore size and volume, carbon load, and end-capping can lead to significant differences in retention times, elution profile selectivity, resolution, and recovery. Slight modifications in the gradient profile may be required to achieve equivalency of mapping when using columns from different manufacturers. [NOTE—The equivalency between instrumentation used for the validation of the test and for routine quality control testing should be considered. It might be preferable to use the same HPLC system for all applications. Otherwise, equivalency of the systems is determined, which may require some changes in the chromatographic test conditions.]

Digest Stability—The length of time a digest can be kept before it is chromatographed, as well as the conditions under which the digest is stored before chromatography, is assessed. Several aliquots from a single digest are stored at different storage conditions and chromatographed. These maps are then evaluated for significant differences.

Reproducibility

Determination of various parameters indicated above is repeated using the same USP Reference Standard or Reference Material and test sample in at least two different laboratories by two analysts equipped with similar HPLC systems. The generated peptide maps are evaluated for significant differences.▲

⟨1056⟩ BIOTECHNOLOGY-DERIVED ARTICLES—POLYACRYLAMIDE GEL ELECTROPHORESIS

INTRODUCTION

Scope

Polyacrylamide gel electrophoresis is used for the qualitative characterization of proteins in biological preparations, for control of purity, and for quantitative determinations.

Purpose

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins. Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given in *Validation of the Test* (below).

CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibers and pores that is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerization usually is catalyzed by a free radical–generating system composed of ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel through manipulating the acrylamide concentration, analysts can optimize the resolution of the method for a given protein product. Thus, a given gel is physically characterized by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component of electrophoretic mobility. In the case of proteins, the electrophoretic mobility depends on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration, and the pH of the buffer; by the temperature and the field strength; and by the nature of the support material.

DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000–100,000 Da. It is possible to extend this mass range by various techniques (e.g., gradient gels and particular buffer system). For instance, tricine–sodium dodecyl sulfate (SDS) gels, using tricine instead of glycine (in the method described here) as the trailing ion in the electrophoresis running buffer, can separate very small proteins and peptides under 10,000–15,000 Da.

Denaturing polyacrylamide gel electrophoresis using glycine SDS (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and is the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged, and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS–polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent–polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes migrate toward the anode in a predictable manner; low-molecular-mass complexes migrate faster than larger ones. The molecular mass of a protein therefore can be estimated from its relative mobility in calibrated SDS-PAGE, and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, can change the apparent molecular mass of a protein, because SDS does not bind to a carbohydrate moiety in a manner similar to that of a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.

Depending on the extent of glycosylation and other posttranslational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

Reducing Conditions

Polypeptide subunits and three-dimensional structure often are maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) results in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, analysts can reasonably calculate the molecular mass of the polypeptide by linear regression (or, more closely, by nonlinear regression) in the presence of suitable molecular mass standards.

Nonreducing Conditions

For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS–protein complexes migrate more slowly than their SDS–polypeptide subunits. In addition, nonreduced proteins may not be completely saturated with SDS and hence may not bind the detergent in a constant mass ratio. Moreover, intrachain disulfide bonds constrain the molecular shape, usually in such a way that reduces the Stokes radius of the molecule, thereby reducing the apparent molecular mass, M_r. This makes molecular mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides because it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS

The most popular electrophoretic method for the characterization of complex mixtures of proteins uses a discontinuous buffer system involving two contiguous but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large-volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution and drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed, with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS–protein complexes to form into a thin zone (stack) and to migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS–proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane (Tris) and glycine. Molecular sieving causes the SDS–polypeptide complexes to separate on the basis of their molecular masses.

PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

This section describes the preparation of gels using particular instrumentation. This does not apply to precast gels. For precast gels or any other commercially available equipment, the manufacturer's instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and when the purity of the reagents used is not sufficient, a pretreatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionized with a mixed-bed (anion–cation exchange) resin to remove acrylic acid and other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

Gel Stock Solutions

30% ACRYLAMIDE–BISACRYLAMIDE SOLUTION

Prepare a solution containing 290 g of acrylamide and 10 g of methylenebisacrylamide per liter of water. Filter.

AMMONIUM PERSULFATE SOLUTION

Prepare a small quantity of solution having a concentration of 100 g/L of ammonium persulfate. [NOTE—Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Because ammonium persulfate decomposes rapidly, fresh solutions must be prepared daily.]

TEMED

Use an electrophoresis-grade reagent.

SDS SOLUTION

This is a 100 g/L solution of electrophoresis-grade SDS.

1.5 M BUFFER SOLUTION

Dissolve 90.8 g of Tris in 400 mL of water. Adjust the pH to 8.8 with hydrochloric acid, and dilute to 500.0 mL with water.

1 M BUFFER SOLUTION

Dissolve 60.6 g of Tris in 400 mL of water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 500.0 mL with water.

Assembling the Gel-Molding Cassette

Clean the two glass plates (size, e.g., 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers, and the silicone rubber tubing (e.g., 0.6 mm diameter × 35 cm length) with mild detergent; rinse extensively with water, followed by dehydrated alcohol; and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with nonsilicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer, and follow the long side of the glass plate. While holding the tubing with one finger along the long side, twist the tubing again and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment, and hold the mold together by hand pressure.

Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the gel mold, thus forming the bottom of the gel mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while the clamps were placed. The gel mold is now ready for pouring the gel.

Preparation of the Gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel, because the composition of the two gels in acrylamide–bisacrylamide, buffer, and pH are different.

PREPARATION OF THE RESOLVING GEL

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in *Table 1*. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in *Table 1*, swirl, and pour immediately into the gap between the two glass plates of the mold. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipet, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerization.

Table 1. Preparation of the Resolving Gel

	Component Volume (mL) per Gel Mold Volume Below							
Solution component	**5 mL**	**10 mL**	**15 mL**	**20 mL**	**25 mL**	**30 mL**	**40 mL**	**50 mL**
6% Acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% Acrylamide–Bisacrylamide Solution	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0

Table 1. Preparation of the Resolving Gel *(Continued)*

	Component Volume (mL) per Gel Mold Volume Below							
Solution component	**5 mL**	**10 mL**	**15 mL**	**20 mL**	**25 mL**	**30 mL**	**40 mL**	**50 mL**
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% Acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% Acrylamide–Bisacrylamide Solution	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% Acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% Acrylamide–Bisacrylamide Solution	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% Acrylamide–Bisacrylamide Solution	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
30% Acrylamide–Bisacrylamide Solution	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Buffer Solution	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% Acrylamide–Bisacrylamide Solution	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

PREPARATION OF THE STACKING GEL

After polymerization is complete (about 30 min), pour off the isobutanol, and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in *Table 2*. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in *Table 2*. Swirl, and pour immediately into the gap between the two glass plates of the mold directly onto the surface of the polymerized resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position, and allow it to polymerize at room temperature.

Table 2. Preparation of the Stacking Gel

	Component Volume (mL) per Gel Mold Volume Below							
Solution component	**1 mL**	**2 mL**	**3 mL**	**4 mL**	**5 mL**	**6 mL**	**8 mL**	**10 mL**
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% Acrylamide–Bisacrylamide Solution	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Buffer Solution	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
SDS Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
Ammonium Persulfate Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Preparation of the Sample

Unless otherwise specified in the specific monograph, the samples can be prepared as follows:

SDS-PAGE SAMPLE BUFFER (CONCENTRATED)

Dissolve 1.89 g of Tris, 5.0 g of sodium lauryl sulfate, and 50 mg of bromophenol blue in water. Add 25.0 mL of glycerol, and dilute to 100 mL with water. Adjust the pH to 6.8 with hydrochloric acid, and dilute to 125 mL with water.

SDS-PAGE SAMPLE BUFFER FOR REDUCING CONDITIONS (CONCENTRATED)

Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Add 25.0 mL of 2-ME. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Alternatively, DTT can be used as reducing agent instead of 2-ME. In this case prepare the sample buffer as follows: Dissolve 3.78 g of Tris, 10.0 g of SDS, and 100 mg of bromophenol blue in water. Add 50.0 mL of glycerol, and dilute to 200 mL with water. Adjust to pH 6.8 with hydrochloric acid, and dilute to 250.0 mL with water. Immediately before use, add DTT to a final concentration of 100 mM.

SDS-PAGE RUNNING BUFFER

Dissolve 151.4 g of Tris, 721.0 g of glycine, and 50.0 g of sodium lauryl sulfate in water, and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with water, and mix. Measure the pH of the diluted solution. The pH is between 8.1 and 8.8.

SAMPLE SOLUTION (NONREDUCING CONDITIONS)

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer (Concentrated)*.

SAMPLE SOLUTION (REDUCING CONDITIONS)

Mix equal volumes of: a mixture comprising water plus the preparation or the reference solutions, and *SDS-PAGE Sample Buffer for Reducing Conditions (Concentrated)* containing 2-ME (or DTT) as the reducing agent.

The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: Keep for 5 min in a boiling water bath or in a block heater set at 100°, and then chill. (Note that temperature and time may vary in the monograph because protein cleavage may occur during the heat treatment.)

Mounting the Gel in the Electrophoresis Apparatus and Electrophoretic Separation

After polymerization is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE Running Buffer* to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing, and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, because this will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse each well with *SDS-PAGE Running Buffer*. Prepare the test and reference solutions in the recommended sample buffer, and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells.

Start the electrophoresis using the conditions recommended by the equipment manufacturer. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness, and electrophoresis running time and current or voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is near the bottom of the gel stop the electrophoresis. Remove the gel assembly from the apparatus, and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel, and immediately proceed with staining.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS—GRADIENT CONCENTRATION GELS

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient-forming apparatus. Ready-to-use gradient gels are commercially available with specific recommended protocols.

Gradient gels offer some advantages over fixed-concentration gels. Some proteins that co-migrate on fixed-concentration gels can be resolved within gradient gels. During electrophoresis the proteins migrate until the pore size stops further progress, and therefore a stacking effect occurs, resulting in sharper bands. According to *Table 3*, gradient gels also allow separation of a wider range of protein molecular masses than do single, fixed-concentration gels.

Table 3 gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular ranges. Note that other gradient shapes (e.g., concave) can be prepared for specific applications.

Table 3. Acrylamide Gradient Percentages Recommended for Expected Protein Molecular Weights

Acrylamide (%)	Protein Range (kDa)
5–15	20–250
5–20	10–200
10–20	10–150
8–20	8–150

Gradient gels also are used for molecular mass determination and protein purity determination.

DETECTION OF PROTEINS IN GELS

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods, and commercial kits are available. For example, fluorescent stains are visualized using a fluorescent imager and often provide a linear response over a wide range of protein concentrations—often several orders of magnitude, depending on the protein.

Coomassie staining has a protein detection level of approximately 1–10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels, and a band containing 10–100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of one or two orders of magnitude has been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining, but the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e., when the analyst deems the staining satisfactory. Wide dynamic ranges of reference proteins are important to use because they help assess the intra-experimental sensitivity and linearity. All gel-staining steps are done while wearing gloves, at room temperature, with gentle shaking (e.g., on an orbital shaker platform), and using any convenient container.

Staining Reagents

DESTAINING SOLUTION

Prepare a mixture of 1 volume of glacial acetic acid, 4 volumes of methanol, and 5 volumes of water.

COOMASSIE STAINING SOLUTION

Prepare a 1.25 g/L solution of acid blue 83 in *Destaining Solution*. Filter.

FIXING SOLUTION

To 250 mL of methanol, add 0.27 mL of formaldehyde, and dilute to 500.0 mL with water.

SILVER NITRATE REAGENT

To a mixture of 3 mL of concentrated ammonia and 40 mL of 1 M sodium hydroxide, add 8 mL of a 200 g/L solution of silver nitrate, dropwise, with stirring. Dilute to 200 mL with water.

DEVELOPER SOLUTION

Dilute 2.5 mL of a 20 g/L solution of citric acid and 0.27 mL of formaldehyde to 500.0 mL with water.

BLOCKING SOLUTION

A 10% (v/v) solution of acetic acid.

Coomassie Staining

Immerse the gel in a large excess of *Coomassie Staining Solution*, and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *Destaining Solution*. Change the *Destaining Solution* several times until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. More rapid destaining can be achieved by including a few grams of anion-exchange resin or a small sponge in the *Destaining Solution*. [NOTE—The acid–alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid, 4 volumes of methanol, and 5 volumes of water for 1 h before it is immersed in the *Coomassie Staining Solution*.]

Silver Staining

Immerse the gel in a large excess of *Fixing Solution*, and allow it to stand for 1 h. Remove the *Fixing Solution*, add fresh *Fixing Solution*, and incubate for at least 1 h or overnight, if convenient. Discard the *Fixing Solution*, and wash the gel in a large excess of water for 1 h. Soak the gel for 15 min in a 1% (v/v) solution of glutaraldehyde. Wash the gel twice for 15 min in a large excess of water. Soak the gel in fresh *Silver Nitrate Reagent* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of water. Immerse the gel for about 1 min in *Developer Solution* until satisfactory staining has been obtained. Stop the development by incubation in the *Blocking Solution* for 15 min. Rinse the gel with water.

RECORDING THE RESULTS

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, gel-scanning systems with data analysis software are commercially available to photograph and analyze the wet gel immediately.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of glycerol for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/L solution of glycerol.

Drying of stained SDS polyacrylamide gels is one of the methods to have permanent documentation. This method frequently results in gel cracking during drying between cellulose films.

Immerse two sheets of porous cellulose film in water, and incubate for 5–10 min.

Place one of the sheets on a drying frame. Carefully lift the gel, and place it on the cellulose film. Remove any trapped air bubbles, and pour a few mL of water around the edges of the gel. Place the second sheet on top, and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

MOLECULAR MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of prestained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and are loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, mark the position of the bromophenol blue tracking dye to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or R_F. Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_F values. Unknown molecular masses can be estimated by linear regression analysis (more accurately, by nonlinear regression analysis) or interpolation from the curves of log M_r against R_F if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

VALIDATION OF THE TEST

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers, e.g., across 80% of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the R_F. If the plot has a sigmoidal shape, then only data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity also must be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve as a system suitability check of the experiment.

QUANTITATION OF IMPURITIES

SDS-PAGE is often used as a limit test for impurities. When impurities are quantitated by normalization to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein, as described in the introduction of the section *Detection of Proteins in Gels*, the linear range can vary but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentrations.

When the impurity limit is specified in the individual monograph, analysts should prepare a reference solution corresponding to that level of impurity by diluting the test solution. For example, when the limit is 5%, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities can be quantified by normalization to the main band, using an integrating densitometer, or by image analysis.

⟨1057⟩ BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are provided in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.

INTRODUCTION

The following procedures are provided as illustrations of the determination of total protein content in pharmacopeial preparations. Other techniques, such as HPLC, are also acceptable if total protein recovery is demonstrated. Many of the total pro-

tein assay methods described below can be performed successfully using kits from commercial sources. [NOTE—Where water is required, use distilled water.]

Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of *Method 1*. Protein determination at 280 nm is mainly a function of the tyrosine and tryptophan content of the protein. If the buffer used to dissolve the protein has a high absorbance relative to that of water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation. [NOTE—Keep the *Test Solution*, the *Standard Solution*, and the buffer at the same temperature during testing.]

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2 to 2 mg per mL.

STANDARD SOLUTION

Unless otherwise specified in the individual monograph, prepare a solution of USP Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the *Test Solution*.

PROCEDURE

Concomitantly determine the absorbances of the *Standard Solution* and the *Test Solution* in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), using the buffer as the blank. To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

LIGHT-SCATTERING

The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the test specimen. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light-scattering, determine the absorbances of the *Test Solution* at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light-scattering at 280 nm. Subtract the absorbance due to light-scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-µm porosity or clarification by centrifugation may be performed to reduce the effect of light-scattering, especially if the solution is noticeably turbid.

CALCULATIONS

Calculate the concentration, C_U, of protein in the test specimen by the formula:

$$C_S(A_U/A_S)$$

in which C_S is the concentration of the *Standard Solution;* and A_U and A_S are the corrected absorbances of the *Test Solution* and the *Standard Solution,* respectively (see ⟨857⟩).

Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic–tungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. Where separation of interfering substances fom the protein in the test specimen is necessary, proceed as directed below for *Interfering Substances* prior to preparation of the *Test Solution.* The effect of interfer-

ing substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement.

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution.* Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 5 and 100 µg of protein per mL, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions.* An appropriate buffer will produce a pH in the range of 10.0 to 10.5.

BLANK

Use the buffer used for the *Test Solution* and the *Standard Solutions.*

REAGENTS AND SOLUTIONS

Copper Sulfate Reagent—Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.

SDS Solution—Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.

Sodium Hydroxide Solution—Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.

Alkaline Copper Reagent—Prepare a mixture of *Copper Sulfate Reagent, SDS Solution,* and *Sodium Hydroxide Solution* (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.

Diluted Folin-Ciocalteu's Phenol Reagent—Mix 10 mL of Folin-Ciocalteu's phenol TS with 50 mL of water. Store in an amber bottle, at room temperature.

PROCEDURE

To 1 mL of each *Standard Solution,* the *Test Solution,* and the *Blank,* add 1 mL of *Alkaline Copper Reagent,* and mix. Allow to stand at room temperature for 10 minutes. Add 0.5 mL of the *Diluted Folin-Ciocalteu's Phenol Reagent* to each solution, mix each tube immediately, and allow to stand at room temperature for 30 minutes. Determine the absorbances of the solutions from the *Standard Solutions* and the *Test Solution* at the wavelength of maximum absorbance at 750 nm with a suitable spectrophotometer (see 〈857〉), using the solution from the *Blank* to set the instrument to zero.

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test Solution,* determine the concentration of protein in the *Test Solution.*

INTERFERING SUBSTANCES

In the following procedure, deoxycholate–trichloroacetic acid is added to a test specimen to remove interfering substances by precipitation of proteins before testing. This technique also can be used to concentrate proteins from a dilute solution.

Sodium Deoxycholate Reagent—Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

Trichloroacetic Acid Reagent—Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.

Procedure—Add 0.1 mL of *Sodium Deoxycholate Reagent* to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 minutes. Add 0.1 mL of *Trichloroacetic Acid Reagent*, and mix on a vortex mixer. Centrifuge at 3000 × *g* for 30 minutes, decant the liquid, and remove any residual liquid with a pipet. Redissolve the protein pellet in 1 mL of *Alkaline Copper Reagent.* Proceed as directed for the *Test Solution.*

NOTE—Color development reaches a maximum in 20 to 30 minutes during incubation at room temperature, after which there is a gradual loss of color. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the brilliant blue G dye binds to protein. The brilliant blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins.

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution.* Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 100 µg and 1 mg of protein per mL, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions.*

BLANK

Use the buffer used to prepare the *Test Solution* and the *Standard Solutions.*

COOMASSIE REAGENT

Dissolve 100 mg of brilliant blue G* in 50 mL of alcohol. [NOTE—Not all dyes have the same brilliant blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman No. 1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [NOTE—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

PROCEDURE

Add 5 mL of the *Coomassie Reagent* to 100 µL of each *Standard Solution,* the *Test Solution,* and the *Blank,* and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the solutions from the *Standard Solutions* and the *Test Solution* at 595 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument to zero. [NOTE—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.]

There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test Solution,* determine the concentration of protein in the *Test Solution.*

Method 4

This method, commonly referred to as the bicinchoninic acid or BCA assay, is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{1+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement.

* Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, Hauppauge, NY) is an acceptable grade.

General Chapters

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution.* Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 10 and 1200 µg of protein per mL, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions.*

BLANK

Use the buffer used to prepare the *Test Solution* and the *Standard Solutions.*

REAGENTS

BCA Reagent—Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.

Copper Sulfate Reagent—Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.

Copper–BCA Reagent—Mix 1 mL of *Copper Sulfate Reagent* and 50 mL of *BCA Reagent.*

PROCEDURE

Mix 0.1 mL of each *Standard Solution,* the *Test Solution,* and the *Blank* with 2 mL of the *Copper–BCA Reagent.* Incubate the solutions at 37° for 30 minutes, note the time, and allow to come to room temperature. Within 60 minutes following the incubation time, determine the absorbances of the solutions from the *Standard Solutions* and the *Test Solution* in quartz cells at 562 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature. If substances that will cause interference in the test are present, proceed as directed for *Interfering Substances* under *Method 2.* Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test Solution,* determine the concentration of protein in the *Test Solution.*

Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm.

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, prepare a solution of Albumin Human for which the protein content has been previously determined by nitrogen analysis (using the nitrogen-to-protein conversion factor of 6.25) or of the USP Reference Standard or reference material for the protein under test in sodium chloride solution (9 in 1000). Dilute portions of this solution with sodium chloride solution (9 in 1000) to obtain not fewer than three *Standard Solutions* having concentrations between 0.5 and 10 mg per mL, the concentrations being evenly spaced. [NOTE—Low responses may be observed if the sample under test has a significantly different level of proline than that of Albumin Human. A different standard protein may be employed in such cases.]

TEST SOLUTION

Prepare a solution of the test protein in sodium chloride solution (9 in 1000) having a concentration within the range of the concentrations of the *Standard Solutions.*

BLANK

Use sodium chloride solution (9 in 1000).

BIURET REAGENT

Dissolve about 3.46 g of cupric sulfate in 10 mL of hot water, and allow to cool (*Solution 1*). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of sodium carbonate in 80 mL of hot water, and allow to cool (*Solution 2*). Mix *Solution 1* and *Solution 2,* and dilute with water to 200 mL. This *Biuret Reagent* is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

PROCEDURE

To one volume of a solution of the *Test Solution* add an equal volume of sodium hydroxide solution (6 in 100), and mix. Immediately add a volume of *Biuret Reagent* equivalent to 0.4 volume of the *Test Solution,* and mix. Allow to stand at a temperature between 15° and 25° for not less than 15 minutes. Within 90 minutes after the addition of the *Biuret Reagent,* determine the absorbances of the *Standard Solutions* and the solution from the *Test Solution* at the wavelength of maximum absorbance at 545 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument to zero. [NOTE—Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

CALCULATIONS

Using the least-squares linear regression method, plot the absorbances of the *Standard Solutions* versus the protein concentrations, determine the standard curve best fitting the plotted points, and calculate the correlation coefficient for the line. [NOTE—Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear.] A suitable system is one that yields a line having a correlation coefficient of not less than 0.99. From the standard curve and the absorbance of the *Test Solution,* determine the concentration of protein in the test specimen, making any necessary correction.

INTERFERING SUBSTANCES

To minimize the effect of interfering substances, the protein can be precipitated from the initial test specimen as follows. Add 0.1 volume of 50% trichloroacetic acid to 1 volume of a solution of the test specimen, withdraw the supernatant layer, and dissolve the precipitate in a small volume of 0.5 N sodium hydroxide. Use the solution so obtained to prepare the *Test Solution.*

COMMENTS

This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the *Biuret Reagent* as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the *Biuret Reagent* will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg per mL.

Method 6

This fluorometric method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the *o*-phthalaldehyde reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with *o*-phthalaldehyde and must be avoided or removed. Ammonia at high concentrations will react with *o*-phthalaldehyde as well. The fluorescence obtained when amine reacts with *o*-phthalaldehyde can be unstable. The use of automated procedures to standardize this procedure may improve the accuracy and precision of the test.

General Chapters

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution.* Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 10 and 200 µg of protein per mL, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions.*

BLANK

Use the buffer used to prepare the *Test Solution* and the *Standard Solutions.*

REAGENTS

Borate Buffer—Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.

Stock OPA Reagent—Dissolve about 120 mg of *o*-phthalaldehyde in 1.5 mL of methanol, add 100 mL of *Borate Buffer,* and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

OPA Reagent—To 5 mL of *Stock OPA Reagent* add 15 µL of 2-mercaptoethanol. Prepare at least 30 minutes prior to use. This reagent is stable for one day.

PROCEDURE

Adjust each of the *Standard Solutions* and the *Test Solution* to a pH between 8 and 10.5. Mix 10 µL of the *Test Solution* and each of the *Standard Solutions* with 100 µL of *OPA Reagent,* and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see *Fluorescence Spectroscopy* 〈853〉), determine the fluorescent intensities of solutions from the *Standard Solutions* and the *Test Solution* at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. [NOTE—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

CALCULATIONS

The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the fluorescent intensity of the *Test Solution,* determine the concentration of protein in the test specimen.

Method 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method. Nitrogen analysis techniques destroy the protein under test but are not limited to protein presentation in an aqueous environment.

PROCEDURE 1

Determine the nitrogen content of the protein under test as directed under *Nitrogen Determination* 〈461〉. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

PROCEDURE 2

Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°), which produces nitric oxide (NO) and similar oxides of nitrogen (NO_x) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas,

which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

CALCULATIONS

The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the USP Reference Standard or reference material.

⟨1058⟩ ANALYTICAL INSTRUMENT QUALIFICATION

INTRODUCTION

A large variety of laboratory equipment, instruments, and computerized analytical systems, ranging from simple nitrogen evaporators to complex multiple-function technologies (see *Instrument Categories*), are used in the pharmaceutical industry to acquire data to help ensure that products are suitable for their intended use. An analyst's objective is to consistently obtain reliable and valid data suitable for the intended purpose. Depending on the applications, users validate their procedures, calibrate their instruments, and perform additional instrument checks, such as system suitability tests and analysis of in-process quality control check samples to help ensure that the acquired data are reliable. With the increasing sophistication and automation of analytical instruments, an increasing demand has been placed on users to qualify their instruments.

Unlike method validation and system suitability activities, analytical instrument qualification (AIQ) currently has no specific guidance or procedures. Competing opinions exist regarding instrument qualification and validation procedures and the roles and responsibilities of those who perform them. Consequently, various approaches have been used for instrument qualification, approaches that require varying amounts of resources and generate widely differing amounts of documentation. This chapter provides a scientific approach to AIQ and considers AIQ as one of the major components required for generating reliable and consistent data. Note that the amount of rigor applied to the qualification process will depend on the complexity and intended use of the instrumentation. This approach emphasizes AIQ's place in the overall process of obtaining reliable data from analytical instruments.

Validation versus Qualification

In this chapter, the term validation is used for manufacturing processes, analytical procedures, and software procedures and the term qualification is used for instruments. Thus, the phrase "analytical instrument qualification" (AIQ) is used for the process of ensuring that an instrument is suitable for its intended application.

COMPONENTS OF DATA QUALITY

There are four critical components involved in the generation of reliable and consistent data (quality data). *Figure 1* shows these components as layered activities within a quality triangle. Each layer adds to the overall quality. Analytical instrument qualification forms the base for generating quality data. The other components essential for generating quality data are analytical method validation, system suitability tests, and quality control check samples. These quality components are described below.

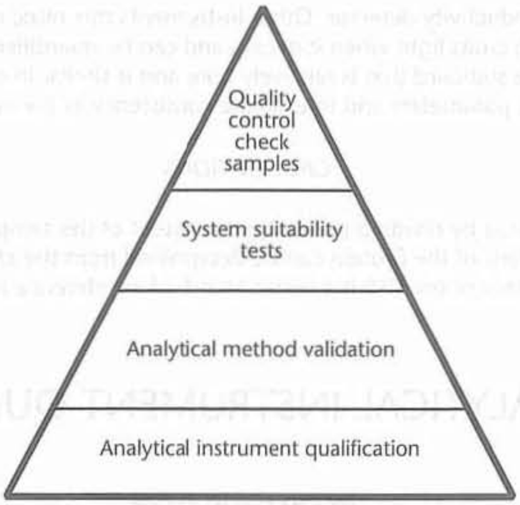


Figure 1. Components of data quality.

Analytical Instrument Qualification

AIQ is the collection of documented evidence that an instrument performs suitably for its intended purpose. Use of a qualified instrument in analyses contributes to confidence in the validity of generated data.

Analytical Method Validation

Analytical method validation is the collection of documented evidence that an analytical procedure is suitable for its intended use. Use of a validated procedure with qualified analytical instruments provides confidence that the procedure will generate test data of acceptable quality. Additional guidance on validation of compendial procedures may be found in the general information chapter *Validation of Compendial Procedures* 〈1225〉.

System Suitability Tests

System suitability tests verify that the system will perform in accordance with the criteria set forth in the procedure. These tests are performed along with the sample analyses to ensure that the system's performance is acceptable at the time of the test. USP general chapter *Chromatography* 〈621〉 presents a more detailed discussion of system suitability tests as related to chromatographic systems.

Quality Control Check Samples

Many analysts carry out their tests on instruments standardized using reference materials and/or calibration standards. Some analyses also require the inclusion of quality control check samples to provide an in-process or ongoing assurance of the test's suitable performance. In this manner, AIQ and analytical method validation contribute to the quality of analysis *before* analysts conduct the tests. System suitability tests and quality control checks help ensure the quality of analytical results *immediately before* or *during* sample analysis.

ANALYTICAL INSTRUMENT QUALIFICATION PROCESS

The following sections address in detail the AIQ process. The other three components of building quality into analytical data—analytical method validation, system suitability tests, and quality control check samples—are not within the scope of this chapter.

Qualification Phases

Instrument qualification is not a single continuous process, but instead results from several discrete activities. For convenience, these activities can be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

Some AIQ activities cover more than one qualification phase, and analysts potentially could perform them during more than one of the phases (see *Table 1*). However, in many instances there is need for specific order to the AIQ activities; for example,

installation qualification must occur first in order to initiate other qualification activities. The AIQ activities will be defined and documented.

Table 1. Timing, Applicability, and Activities for Each Phase of Analytical Instrument Qualification*

Design Qualification	Installation Qualification		Operational Qualification		Performance Qualification
Timing and Applicability					
Prior to purchase of a new model of instrument	At installation of each instrument (new, old, or existing unqualified)		After installation or major repair of each instrument		Periodically at specified intervals for each instrument
Activities					
Assurance of manufacturer's DQ	Description	⇆	Fixed parameters		Preventive maintenance and repairs
Assurance of adequate support availability from manufacturer	Instrument delivery				Establish practices to address operation, calibration, maintenance, and change control
Instrument's fitness for use in laboratory	Utilities/facility	⇆	Environment		
	Assembly and installation				
	Network and data storage	⇆	Secure data storage, backup, and archive		
	Installation verification	⇆	Instrument function tests	⇆	Performance checks

* Activities under each phase are usually performed as given in the table. However, in some cases, it may be more appropriate to perform or combine a given activity with another phase. Such activities spanning more than one qualification phase are shown as connected by double arrows. If an activity listed under a given phase is performed under another phase, it is not necessary to repeat the activity under the phase where the activity is listed. Performing the activity is far more important than the phase under which the activity is performed.

DESIGN QUALIFICATION

Design qualification (DQ) is the documented collection of activities that define the functional and operational specifications of the instrument and criteria for selection of the vendor, based on the intended purpose of the instrument. Design qualification (DQ) may be performed not only by the instrument developer or manufacturer but also may be performed by the user. The manufacturer is generally responsible for robust design and maintaining information describing how the analytical instrument is manufactured (design specifications, functional requirements, etc.) and tested before shipment to users. Nonetheless, the user should ensure that commercial off-the-shelf (COTS) instruments are suitable for their intended application and that the manufacturer has adopted a quality system that provides for reliable equipment. Users should also determine the manufacturer's capability for support installation, services, and training. This determination might be aided by the user's previous interaction with the manufacturer.

INSTALLATION QUALIFICATION

Installation qualification (IQ) is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, and is properly installed in the selected environment, and that this environment is suitable for the instrument. IQ applies to an instrument that is new or was pre-owned, or to any instrument that exists on site but has not been previously qualified. Relevant parts of IQ would also apply to a qualified instrument that has been transported to another location or is being reinstalled for other reasons, such as prolonged storage. The activities and documentation typically associated with IQ are as follows.

Description—Provide a description of the instrument or the collection of instrument components, including its manufacturer, model, serial number, software version, and location. Use drawings and flow charts where appropriate.

Instrument Delivery—Ensure that the instrument, software, manuals, supplies, and any other instrument accessories arrive as specified in the purchase order and that they are undamaged. For a pre-owned or existing instrument, manuals and documentation should be obtained.

Utilities/Facility/Environment—Verify that the installation site satisfactorily meets manufacturer-specified environmental requirements.

Assembly and Installation—Assemble and install the instrument, and perform any preliminary diagnostics and testing. Assembly and installation may be done by the manufacturer, vendor, specialized engineers, or qualified in-house personnel. Manufacturer-established installation tests and guides provide a valuable baseline reference for determining instrument acceptance. Any abnormal event observed during assembly and installation merits documenting. Installation packages purchased from the manufacturer or the vendor may, however, need to be supplemented with user-specific criteria.

Network and Data Storage—Some analytical systems require users to provide network connections and data storage capabilities at the installation site. When required, connect the instrument to the network, and check its functionality.

Installation Verification—Perform the initial diagnostics and testing of the instrument after installation.

OPERATIONAL QUALIFICATION

After a successful IQ, the instrument is ready for OQ testing. Operational qualification (OQ) is the documented collection of activities necessary to demonstrate that an instrument will function according to its operational specification in the selected environment. Testing activities in the OQ phase may consist of these test parameters.

Fixed Parameters—These tests measure the instrument's nonchanging parameters such as length, height, weight, voltage inputs, acceptable pressures, and loads. If the manufacturer-supplied specifications for these parameters satisfy the user, the test requirements may be waived. However, if the user wants to confirm the parameters, testing can be performed at the user's site. Fixed parameters do not change over the life of the instrument, and therefore never need redetermination. [NOTE—These tests could also be performed during the IQ phase (see *Table 1*); if so, fixed parameters need not be redetermined as part of OQ testing.]

Secure Data Storage, Backup, and Archiving—When applicable, test secure data handling such as storage, backup, audit trails, and archiving at the user's site according to written procedures.

Instrument Function Tests—Instrument functions required by the user should be tested to verify that the instrument operates as intended by the manufacturer. Manufacturer-supplied information is useful in identifying specifications for these parameters and in designing tests to evaluate the identified parameters. Users, or their qualified designees, should perform these tests to verify that the instrument meets manufacturer or user specifications in the user's environment.

The extent of OQ testing that an instrument undergoes depends on its intended applications. Therefore, no specific OQ tests for any instrument or application are offered in this chapter.

Routine analytical tests do not constitute OQ testing. OQ tests are specifically designed to verify the instrument's operation according to specifications in the user's environment, and repeating the testing at regular intervals may not be required. However, when the instrument undergoes major repairs or modifications, relevant OQ and/or PQ tests should be repeated to verify whether the instrument continues to operate satisfactorily. If an instrument is moved to another location, an assessment should be made of what, if any, OQ test should be repeated.

OQ tests can be modular or holistic. Modular testing of individual components of a system may facilitate interchanging of such components without requalification. Holistic tests, which involve the entire system, are also acceptable.

PERFORMANCE QUALIFICATION

Performance qualification (PQ) is the documented collection of activities necessary to demonstrate that an instrument consistently performs according to the specifications defined by the user, and is appropriate for the intended use. After IQ and OQ have been performed, the instrument's continued suitability for its intended use is demonstrated through performance qualification. The PQ phase may include the following parameters.

Performance Checks—Set up a test or series of tests to verify the acceptable performance of the instrument for its intended use. PQ tests are usually based on the instrument's typical on-site applications and may consist of analyzing known components or standards. The tests should be based on good science and reflect the general intended use of the instrument. Some system suitability tests or quality control checks that are performed concurrently with the test samples can be used to demonstrate that the instrument is performing suitably. PQ tests may resemble those performed during OQ, but the specifications for their results may be set differently if required. Nevertheless, user specifications for PQ tests should demonstrate trouble-free instrument operation for the intended applications. As is the case with OQ testing, PQ tests may be modular or holistic.

Testing frequency depends on the ruggedness of the instrument and the criticality of the tests performed. Testing may be unscheduled—for example, each time the instrument is used. It may also be scheduled for regular intervals. Experience with the instrument can influence this decision. It may be useful to repeat the same PQ tests each time the instrument is used so that a history of the instrument's performance can be compiled. Alternatively, the instrument may be incorporated into an integrated support system to assure that it remains continually qualified. Some system suitability tests or quality control checks that are performed concurrently with the test samples also imply that the instrument is performing suitably.

Preventive Maintenance and Repairs—When an instrument fails to meet PQ test specifications, it requires maintenance or repair. A periodic preventive maintenance may also be recommended for many instruments. The relevant PQ test(s) should be repeated after the needed maintenance or repair to ensure that the instrument remains qualified.

Practices for Operation, Calibration, Maintenance, and Change Control—Establish practices to maintain and calibrate the instrument. Each maintenance and calibration activity should be documented.

ROLES AND RESPONSIBILITIES

Users

Users are ultimately responsible for instrument operations and data quality. The user's group encompasses analysts, their supervisors, instrument specialists, and organization management. Users should be adequately trained in the instrument's use, and their training records should be maintained as required by the regulations.

Users should also be responsible for qualifying their instruments because their training and expertise in the use of instruments make them the best-qualified group to design the instrument test(s) and specification(s) necessary for successful AIQ. Consultants, equipment manufacturer or vendors, validation specialists, and quality assurance (QA) personnel can advise and assist as needed, but the final responsibility for qualifying instruments lies with the users. The users must also maintain the instrument in a qualified state by routinely performing PQ.

Quality Unit

The role of the Quality Unit in AIQ remains the same as for any other regulated activity. Quality personnel are responsible for assuring that the AIQ process meets compliance requirements, that processes are being followed, and that the intended use of the equipment is supported by valid and documented data.

Manufacturers

Manufacturers and developers are responsible for DQ when designing the instrument. They are also responsible for validation of relevant processes used in manufacturing and assembly of the instrument. Manufacturers should test the assembled instruments before shipping them to users.

Finally, it is desirable that manufacturers and vendors should notify all known users about hardware defects discovered after a product's release; offer user training, service, repair, and installation support; and invite user audits as necessary.

SOFTWARE VALIDATION

Software used for analytical work can be classified into three categories: firmware; instrument control, data acquisition, and processing software; and stand-alone software. Although software validation is not the primary focus of this chapter, the following sections describe in which cases this activity is under the scope of the analytical instrument qualification.

Firmware

Computerized analytical instruments contain integrated chips with low-level software (firmware). Such instruments will not function without properly operating firmware, and users generally cannot alter firmware design or function. Firmware is therefore considered a component of the instrument itself. Indeed, the qualification of hardware is not possible without operating it via its firmware. Thus, when the hardware (that is, the analytical instrument) is qualified at the user's site, the integrated firmware is also essentially qualified. No separate on-site qualification of the firmware is needed. Whenever possible, the firmware version should be recorded as part of the IQ activities. Any changes made to firmware versions should be tracked through change control of the instrument (see *Change Control,* below).

Instrument Control, Data Acquisition, and Processing Software

Software for instrument control, data acquisition, and processing for many of today's computerized instruments is loaded on a computer connected to the instrument. Operation of the instrument is then controlled via the software, leaving fewer operating controls on the instrument. Also, the software is needed for data acquisition and postacquisition calculations. Thus, both hardware and software, their functions inextricably intertwined, are critical to providing analytical results.

The manufacturer should perform DQ, validate this software, and provide users with a summary of validation. At the user site, holistic qualification, which involves the entire instrument and software system, is more efficient than modular validation of the software alone. Thus, the user qualifies the instrument control, data acquisition, and processing software by qualifying the instrument according to the AIQ process.

Stand-Alone Software

An authoritative guide for validating stand-alone software, such as LIMS, is available.[1] The validation process is administered by the software developer, who also specifies the development model appropriate for the software. Validation takes place in a series of activities planned and executed through various stages of the development cycle.

[1] *General Principles of Software Validation: Final Guidance for Industry and FDA Staff,* U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD, January 11, 2002. http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm085281.htm (accessed December 2012.)

CHANGE CONTROL

Changes to instruments, including software, become inevitable as manufacturers add new features and correct known defects. However, implementing all such changes may not always benefit users. Users should therefore adopt changes they deem useful or necessary and should also assess the effects of changes to determine what, if any, requalification is required. The change control process enables them to do this.

Change control may follow the DQ/IQ/OQ/PQ classification process. For DQ, evaluate the changed parameters, and determine whether need for the change warrants implementing it. If implementation of the change is needed, install the changes to the system during IQ. Evaluate which of the existing OQ and PQ tests need revision, deletion, or addition as a result of the installed change. Where the change calls for additions, deletions, or revisions to the OQ or PQ tests, follow the procedure outlined below.

Operational Qualification

Revise OQ tests as necessitated by the change. Perform the relevant tests affected by the change. This ensures the instrument's effective operation after the change is installed.

Performance Qualification

Revise PQ tests as necessitated by the change. Perform the PQ testing after installation of the change if similar testing is not already performed during OQ. In the future, perform the revised PQ testing.

For changes to firmware and to software for instrument control, data acquisition, and processing, change control is performed through DQ/IQ/OQ/PQ of the affected instrument. Change control for stand-alone software requires user-site testing of changed functionality.

AIQ DOCUMENTATION

Documents obtained during instrument qualification should be retained in an accessible manner. Where multiple instruments of one kind exist, documents common to all instruments and documents specific to an instrument may be stored separately. During change control, additional documents may supplement those obtained during the qualification process, and both sets of documents should be retained and maintained in a suitable manner that allows for appropriate protection and access.

INSTRUMENT CATEGORIES

Modern laboratories typically include a suite of instruments and equipment varying from simple nitrogen evaporators to complex automated instruments. Therefore, applying a single set of principles to qualifying such dissimilar instruments would be scientifically inappropriate. Users are most capable of establishing the level of qualification needed for an instrument. On the basis of the level needed, it is convenient to categorize instruments into three groups: A, B, and C, as defined below. Examples of instruments in each group are provided. Note that the list of instruments provided here is for illustration only and is not meant to be exhaustive. It does not provide the exact category for an instrument at a user site. That category should be determined by users for their specific instruments or applications.

The exact grouping of an instrument must be determined by users for their specific requirements. Depending on individual user requirements, the same instrument may appropriately fall into one group for one user and another group for another user. Therefore, a careful selection of groups by users is highly encouraged.

Group A

Group A includes standard equipment with no measurement capability or usual requirement for calibration, where the manufacturer's specification of basic functionality is accepted as user requirements. Conformance of Group A equipment with user requirements may be verified and documented through visual observation of its operation. Examples of equipment in this group are nitrogen evaporators, magnetic stirrers, vortex mixers, and centrifuges.

Group B

Group B includes standard equipment and instruments providing measured values as well as equipment controlling physical parameters (such as temperature, pressure, or flow) that need calibration, where the user requirements are typically the same as the manufacturer's specification of functionality and operational limits. Conformance of Group B instruments or equipment to user requirements is determined according to the standard operating procedures for the instrument or equipment, and documented during IQ and OQ. Examples of instruments in this group are balances, melting point apparatus, light micro-

scopes, pH meters, variable pipets, refractometers, thermometers, titrators, and viscometers. Examples of equipment in this group are muffle furnaces, ovens, refrigerator-freezers, water baths, pumps, and dilutors.

Group C

Group C includes instruments and computerized analytical systems, where user requirements for functionality, operational, and performance limits are specific for the analytical application. Conformance of Group C instruments to user requirements is determined by specific function tests and performance tests. Installing these instruments can be a complicated undertaking and may require the assistance of specialists. A full qualification process, as outlined in this document, should apply to these instruments. Examples of instruments in this group include the following:

- atomic absorption spectrometers
- differential scanning calorimeters
- dissolution apparatus
- electron microscopes
- flame absorption spectrometers
- high-pressure liquid chromatographs
- mass spectrometers
- microplate readers
- thermal gravimetric analyzers
- X-ray fluorescence spectrometers
- X-ray powder diffractometers
- densitometers
- diode-array detectors
- elemental analyzers
- gas chromatographs
- IR spectrometers
- near-IR spectrometers
- Raman spectrometers
- UV/Vis spectrometers
- inductively coupled plasma-emission spectrometers

⟨1059⟩ EXCIPIENT PERFORMANCE

INTRODUCTION

Excipients are used in virtually all drug products and are essential for product manufacturing and performance. Thus, the successful manufacture of a robust product requires the use of well-defined excipients and manufacturing processes that consistently yield a quality product. Excipients used in drug products typically are manufactured and supplied in compliance with compendial standards. However, the effects of excipient properties on the critical quality attributes (CQAs) of a drug product are unique for each formulation and process and may depend on properties of excipients that are not evaluated in *USP* or *NF* monographs. The effects of variations in excipient material attributes depend on the role of an excipient in a formulation and the CQAs of the drug product. This general chapter provides a framework for applying Quality by Design (QBD) principles to excipient quality and performance.

An excipient may be used in different ways or for different purposes in a formulation and may therefore require different material attributes to achieve the desired performance. Excipient functional categories are broad, qualitative, and descriptive terms for the purpose an excipient serves in a formulation. A list of excipients grouped by functional category is included in *NF* under *Front Matter, Excipients,* which summarizes some of the more common purposes that excipients serve in drug products. Also important are the material attributes of the ingredients that must be identified and controlled to ensure the excipient performs its intended function in a drug product. A critical material attribute (CMA) is a physical, chemical, biological, or microbiological property of a material that must be within an appropriate limit, range, or distribution to ensure that drug product CQAs are maintained throughout the product life cycle. Most, but not all, CMAs become tests in a compendial monograph. In some applications, excipient suppliers and users will need to identify and control material attributes in addition to monograph specifications. Identification of CMAs requires a thorough understanding of drug product CQAs; the manufacturing process(es); and the physical, chemical, biological, or microbiological properties of each ingredient. Manufacturers should anticipate lot-to-lot and supplier-to-supplier variability in excipient properties and should have in place appropriate control measures to ensure that CMAs are maintained within the required limits. Prior knowledge, experimental designs, and risk-assessment tools can be used to prioritize and identify CMAs of excipients as well as critical process parameters. A CMA of an excipient

may not be related to the major component of the excipient because, for example, the presence of minor components (e.g., peroxides, elemental impurities, or microbiological content) may affect product stability or quality. Good product development practices, which at times are termed QBD principles, require understanding excipient CMAs that contribute to consistent performance and are the foundation of a control strategy that accommodates excipient variability, consistently achieving final product CQAs.

This informational general chapter provides an overview of the key functional categories of excipients and tests or procedures that can be used to monitor and control CMAs.[1]

In this chapter, the functional categories have been organized by their most typical use in common pharmaceutical dosage forms. However, functional categories can apply to multiple dosage forms. The association of a functional category with a particular dosage form does not limit the use of an excipient to a single type of dosage form or delivery system. Each functional category includes a general description; the mechanisms by which excipients achieve their function; physical properties common to these excipients; chemical properties; and a list of *USP* general chapters that can be useful in the development of specific tests, procedures, and acceptance criteria to ensure that CMAs are adequately monitored and controlled. Because of the complex nature and interplay of formulation ingredients, processing, and dosage form performance requirements, the information provided in this chapter should not be viewed as either restrictive or completely comprehensive.

Change to read:

TABLETS AND CAPSULES

Functional Category: Diluent

DESCRIPTION

Diluents are components that are incorporated into tablet or capsule dosage forms to increase dosage form volume or weight. Sometimes referred to as fillers, diluents often compose a large portion of the dosage form, and the quantity and type of diluent selected often depend on its physical and chemical properties. Thus, successful and robust manufacturing and dosage form performance depend on the measurement and control of the CMAs.

FUNCTIONAL MECHANISM

Among the most important functional roles diluents play is their ability to impart desirable manufacturing properties (e.g., powder flow, tablet compaction strength, wet or dry granule formation, or homogeneity) and performance (e.g., content uniformity, disintegration, dissolution, tablet integrity, friability, or physical and chemical stability). Some diluents (e.g., microcrystalline cellulose) occasionally are referred to as "dry binders" because of the high degree of tablet strength they impart to the final compressed tablet.

PHYSICAL PROPERTIES

The primary physical properties relevant to tablet/capsule diluents are those that can have a direct effect on diluent and formulation performance. These include: 1) particle size and size distribution, 2) particle shape, 3) bulk/tapped/true density, 4) specific surface area, 5) crystallinity, 6) moisture content, 7) powder flow, 8) solubility, 9) crystal form, and 10) compaction properties for tablet dosage forms.

CHEMICAL PROPERTIES

Tablet diluents comprise a large and diverse group of materials that include inorganics (e.g., dibasic calcium phosphate or calcium carbonate), single-component organic materials (e.g., lactose monohydrate or mannitol), and multicomponent (e.g., silicified microcrystalline cellulose or sugar spheres), or complex organics (e.g., microcrystalline cellulose or starch). They may be soluble or insoluble in water, and they may be neutral, acidic, or alkaline in nature. These chemical properties can have a positive or negative effect on the drug substance physical or chemical stability and on performance. Appropriate selection of excipients with desirable physical and chemical properties can enhance the physical and chemical stability as well as the performance of the drug substance and dosage form. The detailed composition of an excipient may be important because excipient function could be influenced by the presence of minor concomitant components that are essential for proper performance. Pharmaceutical scientists may find it necessary to control the presence of undesirable components (e.g., elemental impurities or peroxides) to ensure adequate dosage form stability and performance.

[1] This general information chapter provides nonmandatory information that does not create compendial requirements. For additional information about nonmandatory general chapters and alternative methods and procedures, see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures.*

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in diluent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Powder Fineness* ⟨811⟩, *Specific Surface Area* ⟨846⟩, *Water Determination* ⟨921⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: Wet Binder

DESCRIPTION

Tablet and capsule binders are incorporated into formulations to facilitate the agglomeration of powder into granules during mixing with a granulating fluid such as water, hydroalcoholic mixtures, or other solvents. The binder may be either dissolved or dispersed in the granulation liquid or blended in a dry state, and other components and the granulation liquid may be added separately during agitation. Following evaporation of the granulation liquid, binders typically produce dry granules that achieve the desired properties such as granule size, size distribution, shape, content, mass, and active content. Wet granulation facilitates the further processing of the granules by improving one or more of the granule properties such as flow, handling, strength, resistance to segregation, dustiness, appearance, solubility, compaction, or drug release.

FUNCTIONAL MECHANISM

Binders are soluble or partially soluble in the granulating solvent or, as in the case of native starches, can be made soluble. Concentrated binder solutions also have adhesive properties. Upon addition of liquid, binders typically facilitate the production of moist granules (agglomerates) by altering interparticle adhesion. They also may modify interfacial properties, viscosity, or other properties. During drying they may produce solid bridges that yield improved residual dry granule strength.

PHYSICAL PROPERTIES

Dispersion or dissolution of a binder in the granulation liquid depends on its physical properties: depending on the application, then surface tension, particle size, size distribution, solubility, and viscosity are among the important properties. Homogeneous incorporation of a binder into a dry blend also depends on its physical properties such as particle size, shape, and size distribution. Viscosity often is an important property to consider for binders: for polymers, viscosity is influenced by the nature of the polymer structure, molecular weight, and molecular weight distribution. Polymeric binders may form gels.

CHEMICAL PROPERTIES

Tablet and capsule binders can be categorized as: 1) natural polymers, 2) synthetic polymers, or 3) sugars. The chemical nature of polymers—including polymeric structure, monomer properties and sequence, functional groups, degree of substitution, and cross-linking—influences the complex interactions that can occur during granulation. Natural polymers in particular may exhibit greater variation in their properties because of variations in their sources and therefore their composition.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in binder functions: *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Crystallinity* ⟨695⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Viscosity—Capillary Methods* ⟨911⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: Disintegrant

DESCRIPTION

Disintegrants are functional components that are added to formulations to promote rapid disintegration into smaller units and to allow a drug substance to dissolve more rapidly. Disintegrants are natural, synthetic, or chemically modified natural polymeric substances. When disintegrants come in contact with water or stomach or intestinal fluid, they function by absorbing liquid and start to swell, dissolve, or form gels. This causes the tablet structure to rupture and disintegrate, producing increased surfaces for enhanced dissolution of the drug substance.

FUNCTIONAL MECHANISM(S)

The ability to interact strongly with water is essential to the disintegrant function. Three major mechanisms describe the function of the various disintegrants: volume increase by swelling, deformation, and capillary action (wicking). In tablet formulations, the function of disintegrants is best described as a combination of two or more of these effects. The onset and degree of the locally achieved actions depend on various parameters of a disintegrant, such as its chemical nature and its particle size distribution and particle shape, as well as some important tablet parameters such as hardness and porosity.

PHYSICAL PROPERTIES

The primary physical properties relevant to a disintegrant are those that describe the product's particle structure as a dry powder or its structure when in contact with water. These properties may include: 1) particle size distribution; 2) water absorption rate; 3) swelling ratio or swelling index; and 4) the characterization of the resulting product, whether it is still a particulate or a gel is formed.

CHEMICAL PROPERTIES

Polymers used as disintegrants are either nonionic or anionic with counterions such as sodium, calcium, or potassium. Nonionic polymers are natural or physically modified polysaccharides such as starches, celluloses, pullulan, or cross-linked polyvinylpyrrolidone. The anionic polymers mainly are chemically modified starches, cellulose products, or low-cross-linked polyacrylates. These chemical properties should be considered in the case of ionic polymers. Disintegration performance is affected by pH changes in the gastrointestinal tract or by complex formation with ionic drug substances.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in disintegrant functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: Lubricant

DESCRIPTION

Lubricants typically are used to reduce the frictional forces between particles and between particles and metal-contact surfaces of manufacturing equipment such as tablet punches and dies used in the manufacture of solid dosage forms. Liquid lubricants may be absorbed into the granule matrix before compaction. Liquid lubricants also can be used to reduce metal–metal friction on manufacturing equipment.

FUNCTIONAL MECHANISM

Boundary lubricants function by adhering to solid surfaces (granules and machine parts) and by reducing the particle–particle friction or the particle–metal friction. The orientation of the adherent lubricant particles is influenced by the properties of the substrate surface. For optimal performance, the boundary lubricant particles should be composed of small, plate-like crystals or stacks of plate-like crystals. Fluid film lubricants melt under pressure and thereby create a thin fluid film around particles and on the surface of punches and dies in tablet presses, which helps to reduce friction. Fluid film lubricants resolidify after the pressure is removed. Liquid lubricants are released from the granules under pressure and create a fluid film. They do not resolidify when the pressure is removed but are reabsorbed or redistributed through the tablet matrix over the course of time.

PHYSICAL PROPERTIES

The physical properties that are important for the function of boundary lubricants include particle size, surface area, hydration state, and polymorphic form. Purity (e.g., stearate:palmitate ratio) and moisture content also may be important. The physical properties of possible importance for fluid film lubricants are particle size and solid state/thermal behavior. Purity also may be important.

CHEMICAL PROPERTIES

Lubricants can be classified as boundary lubricants, fluid film lubricants, or liquid lubricants. Boundary lubricants are salts of long-chain fatty acids (e.g., magnesium stearate) or fatty acid esters (e.g., sodium stearyl fumarate) with a polar head and fatty acid tail. Fluid film lubricants are solid fats (e.g., hydrogenated vegetable oil, type 1), glycerides (glyceryl behenate and distea-

rate), or fatty acids (e.g., stearic acid) that melt when subjected to pressure. Liquid lubricants are liquid materials that are released from granules under pressure.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in lubricant functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Thermal Analysis* ⟨891⟩, *Water Determination* ⟨921⟩, and *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩.

ADDITIONAL INFORMATION

Certain lubricants, particularly those used in effervescent dosage forms, do not fall into the chemical categories defined above. These materials are used in special situations, and they are not suitable for universal application. Talc is an inorganic material that may have some lubricant properties. It is generally used in combination with fluid film lubricants to reduce sticking to punches and dies.

Functional Category: Glidant and/or Anticaking Agent

DESCRIPTION

Glidants and anticaking agents are used to promote powder flow and to reduce the caking or clumping that can occur when powders are stored in bulk. In addition, glidants and anticaking agents reduce the incidence of bridging during the emptying of powder hoppers and during powder processing.

FUNCTIONAL MECHANISM

Glidants are thought to work by a combination of adsorption onto the surface of larger particles and reduction of particle–particle adhesive and cohesive forces, thus allowing particles to move more easily relative to one another. In addition, glidants may be dispersed among larger particles and thus may reduce friction between these particles. Anticaking agents may absorb free moisture that otherwise would allow the development of particle–particle bridges that are implicated in caking phenomena.

PHYSICAL PROPERTIES

Primary physical properties of potential importance for glidants and anticaking agents are particle size, particle size distribution, and surface area. They may be slightly hygroscopic.

CHEMICAL PROPERTIES

Glidants and anticaking agents typically are finely divided inorganic materials. Typically they are insoluble in water. Some of these materials are complex.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in glidant or anticaking agent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, and *Water Determination* ⟨921⟩.

Functional Category: Coloring Agent

DESCRIPTION

Coloring agents are incorporated into dosage forms to produce a distinctive appearance that may serve to differentiate a product from others that have a similar physical appearance or, in some instances, to protect photolabile components of the dosage form. These substances are subdivided into dyes (water-soluble substances), lakes (insoluble forms of a dye that result from its irreversible adsorption onto a hydrous metal oxide), inorganic pigments (substances such as titanium dioxide or iron

oxides), and natural colorants (colored compounds not considered dyes per se, such as riboflavin). Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before its use.

The Federal Food, Drug, and Cosmetic Act defines three categories of coloring agents:

- FD&C colors: those certifiable for use in coloring foods, drugs, and cosmetics
- D&C colors: dyes and pigments considered safe in drugs and cosmetics when in contact with mucous membranes or when ingested
- Ext. D&C colors: colorants that, because of their oral toxicity, are not certifiable for use in ingestible products but are considered safe for use in externally applied products.

FUNCTIONAL MECHANISM

Water-soluble dyes usually are dissolved in a granulating fluid for use, although they also may be adsorbed onto carriers such as starch, lactose, or sugar from aqueous or alcoholic solutions. These latter products often are dried and used as formulation ingredients. Because of their insoluble character, lakes almost always are blended with other dry excipients during formulation. For this reason, direct-compression tablets often are colored with lakes.

PHYSICAL PROPERTIES

Particle size and size distribution of dyes and lakes can influence product processing times (blending and dissolution), color intensity, and uniformity of appearance. A coloring agent should be physically nonreactive with other excipients and the drug substances.

CHEMICAL PROPERTIES

The most important properties of a coloring agent are its depth of color and resistance to fading over time. Substances can be graded on their efficiency in reflecting desired colors of visible light, as well as on their molar absorptivities at characteristic wavelengths. A coloring agent should be chemically nonreactive with other excipients and the drug substances. The quality of a coloring agent ordinarily is measured by a determination of its strength, performance, or assay. The impurity profile is established by measurements of insoluble matter, inorganic salt content, metal content, and organic impurities.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected coloring agent functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩ and *Color—Instrumental Measurement* ⟨1061⟩. Instrumental methods should be used to determine the absolute color of a coloring agent.

ADDITIONAL INFORMATION

Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before it is used.

Functional Category: Capsule Shell

DESCRIPTION

The word "capsule" is derived from the Latin *capsula,* which means a small container. Among other benefits, capsules enable pharmaceutical powders and liquids to be formulated for dosing accuracy, as well as ease of transportation. The capsule material should be compatible with all other ingredients in the drug product. Hard capsules typically consist of two parts: both are cylindrical, and one part is slightly longer than the other and is called the body. The cap fits closely on the body to enclose the capsule. In contrast, the soft capsule is a one-piece unit that may be seamed along an axis or may be seamless. The capsule material may be derived from hydrolysis of collagen that originates from porcine, bovine, or fish sources, or it can be of nonanimal origin, e.g., cellulosic or polysaccharide chemical entities. The capsule shell also contains other additives such as plasticizers, colorants, and preservatives. In some cases, capsule shells are sterilized to prevent microbial growth. The capsule shell is an integral part of the formulation, and therefore robust manufacturing and formulation performance depends on the measurement and control of CMAs. Capsules can be used to administer drugs by oral, rectal, vaginal, or inhalation routes.

FUNCTIONAL MECHANISM

Capsules can enclose solid, semisolid, or liquid formulations. Capsules have a variety of benefits: masking unpleasant taste, facilitating blinding in clinical studies, promoting ease of swallowing, and presenting a unique appearance. Conventional cap-

General Chapters

sule shells should dissolve rapidly at 37° in biological fluids such as gastric and intestinal media. However, the solubility properties of the shell can be modified (e.g., with enteric and controlled-release polymers) to control the release of the capsule contents.

PHYSICAL PROPERTIES

The primary physical properties relevant to the capsule shell are those that can have a direct effect on product performance: 1) moisture content, 2) gas permeability, 3) stability on storage, 4) disintegration, 5) compactness, and 6) brittleness. The moisture content varies with the type of capsule. Hard gelatin capsules typically contain 13%–16% water compared to hypromellose (hydroxypropyl methylcellulose or HPMC) capsules that typically contain 4%–7% water content. Moisture content has an important effect on capsule brittleness. Soft gelatin capsules contain 5%–15% water. Equilibrium water content also may be crucial to dosage form stability because water migration can take place between the shell and capsule contents. Gas permeability may be important and generally is greater for HPMC capsules than for gelatin capsules because of the presence of open structures in the former. Unlike HPMC capsules, which do not cross-link, gelatin capsules have the potential to cross-link due to environmental and chemical exposure. Gelatin capsules may undergo cross-linking upon storage at elevated temperature and humidity [e.g., 40°(75% RH)]. Gelatin shell material is also well known to cross-link due to exposure to aldehydes, ketones, and certain dyes in shell formulations. Thus, presence of these materials in excipients should be considered for gelatin encapsulated products. Cross-linking slows in vitro dissolution and often necessitates introduction of enzymes in the test medium. Gelatin capsules should disintegrate within 15 min when exposed to 0.5% hydrochloric acid at 36°–38° but not below 30°. HPMC capsules can disintegrate below 30°.

CHEMICAL PROPERTIES

Gelatin is a commercial protein derived from the native protein, collagen. The product is obtained by partial hydrolysis of collagen derived from skin, white connective tissue, and bones of animals. Type A gelatin is derived by acid treatment, and Type B gelatin is derived from base treatment. The common sources of commercial gelatin are pigskin, cattle hide, cattle bone, cod skin, and tilapia skin. The gelatin capsule shell also typically contains coloring agents, plasticizers such as polyhydric alcohols, natural gums and sugars, and preservatives such as sodium metabisulfite and esters of *p*-hydroxybenzoic acid. The more commonly used nongelatin capsules today are made from HPMC. Different capsule types contain different moisture levels and may thus influence drug product stability. The detailed composition of an excipient may be important because the shell function can be influenced by small amounts of impurities in the excipients (e.g., peroxides in oils or aldehydes in lactose and starches) that can cause capsule cross-linking. The presence in capsule shells of undesirable materials, such as metals, odorants, water-insoluble substances, and sulfur dioxide, should be evaluated to ensure stability and performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Arsenic* ⟨211⟩, ●*Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, ● (Official 1-Jan-2018) *Residue on Ignition* ⟨281⟩, *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Water Determination* ⟨921⟩, and *Color—Instrumental Measurement* ⟨1061⟩.

ADDITIONAL INFORMATION

In addition to the general chapters listed above, useful information for ensuring consistency in selected capsule shell functions may be found in *Gelatin, Gel Strength (Bloom Value)*.

Functional Category: Coating Agent

DESCRIPTION

Oral tablets may be coated using compression coating, sugar coating, or film coating. Compression coating (effectively making a tablet within a tablet) typically uses the same ingredients as a conventional tablet and thus is outside the scope of this section. The term "sugar coating" refers to a process and does not require that sucrose be part of the formulation. Oral capsules can be coated using film-coating procedures. Reasons for coating pharmaceutical dosage forms include masking unpleasant tastes or odors, improving ingestion and appearance, protecting active ingredients from the environment, and modifying the release of the active ingredient (e.g., controlled-release or gastrointestinal targeting). Materials used as coating agents differ depending on the coating process used. Sugar coating was the original coating process. However, today for technical and economic reasons, sugar coating largely has been replaced by film coating. Sugar coating is a complex process that typically involves the application of several different coats including a seal coat, key coat, subcoat, smoothing coat, color coat,

and polishing coat. The coating solutions or suspensions are slowly poured or otherwise applied in aliquots onto a bed of tablets in a slowly rotating pan. The coating process typically takes an extended period (potentially several days) and results in a substantial increase in tablet weight. In contrast, film coating generally is a simpler process in which coating solution or suspension is sprayed onto tablets either in a rotating pan or in a fluid-bed apparatus and results in only a modest increase in capsule or tablet weight. The materials used in both sugar coating and film coating include natural, semisynthetic, and synthetic materials. These may be solutions, suspensions, or colloidal dispersions (latexes or pseudolatexes) that can be applied as either aqueous or nonaqueous systems. In addition, waxes and lipids can be applied as coatings in the molten state without the use of solvents. They also can be applied in the solid state as a polishing coat on top of sugar coating or film coating.

FUNCTIONAL MECHANISM—SUGAR COATING

The seal coat is used to seal the surface of the tablet cores to prevent water in the coating solutions or suspensions from causing the tablet cores to disintegrate during coating. The seal coat typically is a polymer (e.g., shellac) that is insoluble in water and is applied as a thin coat from a solution in a nonaqueous solvent. The key component of the majority of sugar-coating solutions or suspensions is a solute, typically sucrose, that is highly soluble in water and forms a sticky, viscous solution (a syrup) at very high concentration. Other materials can be dissolved or suspended in the solution, depending on the stage during the coating process. As the coating dries, the dissolved coating material adheres to the surface of the tablets. The coating solution or suspension typically is applied in incremental steps, followed by drying, until the requisite coating has been achieved. The key coat is composed of another thin coat that is designed to adhere to the seal-coated cores to provide a base for the subcoat so the latter can adhere to the tablet surface. The subcoat typically contains a high concentration of suspended solids and is designed to increase the weight of the tablets comparatively quickly. The smoothing coat is designed to provide a smooth surface, and the color coat provides the final color if required. Finally, the tablets may be transferred to a polishing pan and polished using a mixture of waxes to provide a high-gloss finish.

FUNCTIONAL MECHANISM—FILM COATING

Film-coating agents are composed of film-forming materials (see *Functional Category: Film-Forming Agent*) that impart desirable pharmaceutical properties such as appearance and patient acceptance (e.g., taste masking and ease of swallowing). Film-coating agents also can serve other functional purposes such as providing a barrier against undesirable chemical reactions or untimely release of a drug from its components. After ingestion, the film coating may dissolve by processes such as hydration, solubilization, or disintegration, depending on the nature of the material used. Enteric coatings are insoluble in acidic (low pH) media but dissolve readily in neutral pH conditions. However, most common film-coating polymers do not have pH-specific solubility. The thickness of the film may vary by application and the nature of the coating agents. In the coating process, the polymer chains spread out on the core surface and coalesce into a continuous film as the solvent evaporates. Polymer solutions or dispersions with a low viscosity and high pigment-binding capacity reduce the coating time and facilitate relatively simple and cost-effective manufacturing. Plastic polymers, waxes, and lipid-based coatings can be applied without solvents by melting and atomization. When molten fluid droplets strike the surface of the fluidized drug particles, they spread and resolidify to form film layers. Therefore, film coating materials generally have the ability to form a complete and stable film around the substrate. The film coating typically is applied uniformly and is carefully dried to ensure that a consistent product is produced. Suitable plasticizers may be required to lower the minimum film-forming temperature of the polymer, and formulators should consider their potential effect on drug release.

PHYSICAL PROPERTIES

Sugar coating is a lengthy, complex process. The physical properties of the seal-coating polymer and solution are important. The physical properties of the syrup component in the subsequent layers and any dissolved or suspended solids also are important, and coating agents must be sufficiently stable during use.

Film coating is a complex process, and the characteristics of the film-forming polymer are important. The particle size of colloidal dispersions varies with their composition and manufacture (latex, pseudolatex, or redispersed powder) and can have an effect on film formation. The surface tension of coating preparations can influence the spray pattern in the manufacturing process. The film should possess sufficient elasticity and mechanical strength to withstand the stresses during coating and packaging operations. For coatings that are applied in a molten state without solvents (plastic polymers, waxes, and lipid-based coatings), melting range and melt viscosity are the primary properties that formulators must consider.

CHEMICAL PROPERTIES

Coating components can be of natural, semisynthetic, or synthetic origin and also can be available in different chemical grades. They comprise a diverse variety of different chemical materials. Formulators must consider the nature of the material and its intended use when they identify and quantitate chemical CMAs to ensure consistent performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected coating agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Dissolution* ⟨711⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩. In addition, the general chapters listed under *Functional Category: Film-Forming Agent* (below) also may be appropriate for the evaluation of film-coating polymers.

ADDITIONAL INFORMATION

Additives often are included in a coating formulation. Fillers (e.g., sugar alcohols, microcrystalline cellulose, calcium carbonate, and kaolin) may be added to increase the solids content of the coating agent without increasing viscosity. Stearic acid can be used to improve the protective function/moisture barrier of a coating. Coloring agents (e.g., titanium dioxide and iron oxides) may be added to modify appearance.

Functional Category: Plasticizer

DESCRIPTION

A plasticizer is a low molecular weight substance that, when added to another material—usually a polymer—makes the latter flexible, resilient, and easier to handle. Plasticizers are key components that determine the physical properties of polymeric pharmaceutical systems such as tablet film coatings and capsule shells.

FUNCTIONAL MECHANISM

Plasticizers function by increasing the intermolecular and intramolecular mobility of the macromolecules that comprise polymeric materials. They achieve this by interfering with the normal intermolecular and intramolecular bonding mechanisms in such systems. The most effective plasticizers exert their effect at low concentrations, typically less than 5% w/w. Plasticizers commonly are added to film coatings (aqueous and nonaqueous systems) and capsule shells (hard and soft varieties) to improve their workability and mechanical ruggedness. Without the addition of plasticizers, such materials can split or fracture prematurely. Plasticizers also are added to semisolid pharmaceutical preparations, such as creams and ointments, to enhance their rheological properties.

PHYSICAL PROPERTIES

The most common plasticizers are low molecular weight (<500 Da) solids or liquids. They typically have low melting points (<100°) and can be volatile (i.e., exert an appreciable vapor pressure) at ambient temperature. Plasticizers can reduce the glass transition temperature (T_g) of the system to which they are added.

CHEMICAL PROPERTIES

Many modern plasticizers are synthetic esters such as citrates and phthalates. Traditional pharmaceutical plasticizers include oils, sugars, and their derivatives.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected excipient functions: *Residual Solvents* ⟨467⟩, *Melting Range or Temperature* ⟨741⟩, *Refractive Index* ⟨831⟩, *Specific Gravity* ⟨841⟩, *Thermal Analysis* ⟨891⟩, and *Water Determination* ⟨921⟩.

ADDITIONAL INFORMATION

The choice of an appropriate plasticizer often is guided by reference to its solubility parameter, which is related to its cohesive energy density. Solubility parameter values for many common materials are tabulated in standard reference texts. To ensure maximum effectiveness, the solubility parameter of the plasticizer and the polymeric system being plasticized should be matched as closely as possible.

Functional Category: Film-Forming Agent

DESCRIPTION

Film-forming agents typically are polymers that can be used to prepare polymer films to coat tablets or capsules for oral administration, to modify appearance, to modify drug release, or to serve other purposes such as melt-in-the-mouth formulations. Polymeric materials used as film-forming agents can be derived from natural, semisynthetic, or synthetic sources, and they can be supplied as powders, granules, pre-prepared solutions, or colloidal dispersions. Colloidal dispersions may contain other components such as plasticizers, surface-active agents, preservatives, or stabilizers. Film-forming agents can be applied as colloidal dispersions (latexes or pseudolatexes) or as aqueous, hydroalcoholic, or nonaqueous polymeric solutions.

FUNCTIONAL MECHANISM

Film-forming agents typically are composed of polymeric materials that possess the ability to form films after solvent evaporation from a solution of the polymer or from the continuous phase of a colloidal dispersion. Thus, the polymer alone must be a solid at ambient temperature and humidity. Some polymers can form films without the inclusion of added components, but other polymers may require the use of additional components such as plasticizers.

PHYSICAL PROPERTIES

Many polymeric film-forming agents are available in a variety of physical grades that typically are based on the nominal viscosity of the particular grade. The physical properties of the polymer usually are those of a solid, and many polymers are available as powders and granules. In addition to the normal properties of bulk powders and granules, other important physical properties of a polymeric film-forming agent are the molecular weight distribution, which is linked to the nominal viscosity of the grade, and the glass transition temperature (T_g). If the film-forming agent is provided as a pre-prepared solution or dispersion, the viscosity of the solution or dispersion can affect performance and should be monitored.

CHEMICAL PROPERTIES

Film-forming agents comprise a diverse group of materials, including natural, semisynthetic, and synthetic materials as discussed above. They may have ionizable functional groups that impart pH-dependent properties and also can be available in different chemical grades (e.g., with different degrees of chemical substitution). Pharmacopeial monographs often describe classes of polymeric materials that allow a considerable range of composition. Formulators should consider these factors when they identify critical material attributes and establish specifications to ensure consistent performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected film-forming agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Dissolution* ⟨711⟩, *Optical Microscopy* ⟨776⟩, *pH* ⟨791⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Bulk Powder Sampling Procedures* ⟨1097⟩, *Near-Infrared Spectroscopy* ⟨1119⟩, *Raman Spectroscopy* ⟨1120⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, *Powder Flow* ⟨1174⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

Functional Category: Flavor and Fragrance

DESCRIPTION

A flavor is a single chemical entity or a blend of chemicals of natural or synthetic origin that has the ability to elicit a taste or aroma (i.e., fragrance) response when orally consumed or smelled. The primary purpose of flavor that is added to a pharmaceutical preparation is to provide all or part of the taste and aroma of the product taken into the mouth. Flavors commonly are used in pharmaceutical oral disintegrating tablets, oral solutions, and oral suspensions to mask objectionable drug taste and to make the formulation more palatable, thus promoting patient compliance.

FUNCTIONAL MECHANISM

Chemicals dissolved in saliva excite chemoreceptors on taste buds that reside primarily on the tongue and thus arouse taste perception. Dissolution also releases volatile chemicals that reach the olfactory receptors, triggering aroma perception. The total of taste and odor responses constitutes flavor. Humans can distinguish among five components of taste: sourness, saltiness, sweetness, bitterness, umami (savory), and a wide range of specific odors. Flavor enhancers and taste modifiers can be used to

modify the sweetness profile of a sweetening agent or to mask off-flavors. For example, organic acids, such as aspartic and glutamic acids, are known to reduce bitterness.

PHYSICAL PROPERTIES

Taste perception depends on physicochemical, physiological, and psychological factors. Physical properties such as particle size, solubility, humectancy, texture, and color all influence the senses. In addition to flavor, the sensory attributes of sight (e.g., appealing color), sound (e.g., crunch of a chewable tablet), and mouth feel (e.g., viscous, slimy, chalky, cloying, or watery) also contribute to and influence the overall sensory experience.

CHEMICAL PROPERTIES

Chemicals that provide one of the five basic tastes possess a wide variety of structures, functional groups, and molecular weights. Chemicals used to flavor pharmaceuticals by providing both odor and taste tend to have low molecular weights (<250 Da) and polar functional groups such as esters, ketones, aldehydes, amines, or alcohols. To increase the stability of the flavor(s) in a solid dosage form and to minimize flavor–drug interactions, formulators can add flavors in an encapsulated or spray-dried form.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in flavor functions: *Light Diffraction Measurement of Particle Size* 〈429〉, *Chromatography* 〈621〉, *Congealing Temperature* 〈651〉, *Loss on Drying* 〈731〉, *Melting Range or Temperature* 〈741〉, *Optical Rotation* 〈781〉, *Particle Size Distribution Estimation by Analytical Sieving* 〈786〉, *Refractive Index* 〈831〉, and *Specific Gravity* 〈841〉.

Functional Category: Release-Modifying Agents

Release-modifying agents are used to control drug release in extended-release formulations (also referred to as prolonged-release or controlled-release formulations). Sustained-release and enteric coating agents are included under *Functional Category: Coating Agent*.

DESCRIPTION

Release-modifying agents change a medicinal product's drug-release pattern to achieve the desired drug plasma profile for a given time. The majority of release-modifying agents are polymers that differ in solubility, ease of erosion, rate of swelling, or sensitivity to the biological environment in which they are placed. These polymers have been used to fabricate matrix- or membrane-based drug delivery systems for oral, parenteral, transdermal, and other routes of administration. Matrix controlled-release drug delivery systems can be classified as hydrophilic eroding matrices, hydrophilic noneroding matrices, or hydrophobic matrices. In membrane controlled-release drug delivery systems, the drug reservoir is coated by a rate-controlling polymeric membrane that may consist of a blend of polymers to control release. Such devices may take the form of tablets, capsules, microspheres, vesicles, fibers, patches, and others. In addition to polymers, certain lipid-based excipients also can be used as release-modifying agents in hydrophobic matrix devices and other types of modified-release systems. Typically, these lipid-based materials are fats and waxes or related materials with melting ranges above 45°.

FUNCTIONAL MECHANISM

Upon contact with a biological fluid, release-controlling polymers may undergo a variety of physical changes such as swelling, gelling, dissolution, or erosion, each of which can be triggered by simple aqueous exposure or can be modulated by pH, osmotic stress, or interactions with bile or other intestinal contents. In addition to physical changes, polymers may undergo chemical degradation by acids, bases, enzymes, water, heat, and others. Any or all of these mechanisms may act in concert to control the rate at which the drug is released from the delivery system.

For hydrophilic matrices in which drug diffusion dominates release rate, the rate of drug release depends on the properties of the polymer gel and the nature of the continuous phase in the interstices of the gel influences the dissolution and diffusion rates of the drug. In the case of eroding matrices, the gel erodes because of the mechanical action of the gastrointestinal tract as the water uptake increases, and the gel becomes more dilute, thus reducing the diffusion distance or releasing drug particles that subsequently dissolve. Hydrophobic matrix-forming materials are not soluble. Drug release from such systems is governed by drug diffusion through the tortuous pores that remain as soluble components dissolve.

Membrane-based drug delivery systems include polymer-coated tablets, capsules, and microspheres. Drug-release mechanisms from such systems are complex and depend on physicochemical characteristics of the drug and polymers or lipids used

as well as biological factors in the case of biocompatible and biodegradable systems. Most commonly, drug release from such systems is governed by drug diffusion through the hydrated rate-controlling membrane.

Other modified-release systems for parenteral use include solid lipid nanoparticles and liposomes. The release mechanisms for these systems often involve a complicated interplay with biological processes such as potential clearance through the reticulo-endothelial system, targeted delivery, and cellular uptake.

Osmotic pump devices are a special case of membrane delivery systems. The rate-controlling polymer is insoluble and semipermeable—i.e., it will allow water but not drug molecules—to diffuse through the membrane. Release is controlled by the osmotic pressure of the core components and the viscosity of the resulting solution or suspension. The drug, either in solution or as a suspension, is forced out of a hole in the membrane, which is typically drilled by a laser during product manufacture.

PHYSICAL PROPERTIES

The physical properties of the release-controlling excipient depend on the chemical type: hydrophilic polymer, hydrophobic polymer, semipermeable polymer blends, or lipid, wax, or biodegradable polymer (which can be hydrophilic or hydrophobic).

Hydrophilic polymers gel in contact with water or aqueous media. Because they should provide resistance to the mechanical action of the gastrointestinal tract during passage, they typically are higher molecular weight grades of the polymers. At the concentrations typically used during in vivo release, these high molecular weight polymers often do not exhibit Newtonian properties except in very dilute solution (if they are soluble). Formulators should monitor the kinetic and viscoelastic properties of the gels formed in the release medium.

Hydrophobic polymers are insoluble in water, and their solution properties are determined in nonaqueous solutions. The polymers used in the preparation of semipermeable membranes in osmotic pump devices also are insoluble in water, and similarly their solution properties are determined in nonaqueous solutions. Similarly, hydrophobic lipid-based materials are insoluble in water.

CHEMICAL PROPERTIES

Release-controlling agents have many different types and origins and are available in a range of grades that reflect the considerable variation in their chemical structures and properties. Formulators must consider these variables during any chemical investigation and when they consider the effects of chemical structure on excipient performance. Properties of interest may include chemical composition for copolymers and cellulosic derivatives, degree of ionization, molecular weight, degree of cross-linking, or, for lipids, fatty acid composition. Residual impurities from the manufacturing process, e.g., monomers, initiators, quenching agents, peroxides, and aldehydes, may affect drug substance stability and should be monitored.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected functions of release-modifying agents: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Dissolution* ⟨711⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Specific Surface Area* ⟨846⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Tensile Strength* ⟨881⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Water Determination* ⟨921⟩, *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩, *Powder Flow* ⟨1174⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

ADDITIONAL INFORMATION

Some release-modifying agents may include additives such as an antioxidant or an anticaking agent.

Change to read:

ORAL LIQUIDS

Functional Category: pH Modifier (Acidifying/Alkalizing/Buffering Agent)

DESCRIPTION

The hydrogen ion concentration, $[H^+]$, in an aqueous solution is expressed as $pH = -\log(H^+)$. The pH of pure water is 7 at 25°. An aqueous solution is acidic at pH <7 and alkaline at pH >7. An acid can be added to acidify a solution. Similarly, a base can be used to alkalize a solution. A buffer is a weak acid (or base) and its salt. When a buffer is present in a solution, the addition of small quantities of strong acid or base leads to only a small change in solution pH. Buffer capacity is influenced by

salt/acid (or base/salt) ratio and total concentration of acid (or base) and salt. The pH of pharmaceutical solutions typically is controlled using acidifying/alkalizing and buffering agents to: 1) maintain a pH close to that of relevant body fluid to avoid irritation, 2) improve drug stability where it is found to be pH dependent, 3) control equilibrium solubility of weak acids or bases, and 4) maintain a consistent ionization state of molecules during chemical analysis, e.g., high-performance liquid chromatography.

FUNCTIONAL MECHANISM

The ionization equilibria of weak bases, weak acids, and water are the key to the functions of acidifying, alkalizing, and buffering agents. The autoprotolytic reaction of water can be expressed as:

$$H_2O + H_2O \leftrightarrow H_3O^+ + OH^-$$

The autoprotolysis constant (or ion product) of water is $K_w = 1 \times 10^{-14}$ at 25° and varies significantly with temperature. Because the concentrations of hydrogen and hydroxyl ions in pure water are equal, each has the value of approximately 1×10^{-7} mol/L, leading to the neutral pH of 7 at 25°. When an acid, base, or salt of a weak acid (or base) is added, the ionization equilibrium of water is shifted so that $[H^+][OH^-]$ remains constant, thus resulting in a solution pH that is different from 7.

PHYSICAL PROPERTIES

pH modifiers typically are dissolved in liquid dosage forms. Physical properties may be important and should be considered because they may influence processing requirements (e.g., particle size may influence mixing time required to dissolve a pH modifier).

CHEMICAL PROPERTIES

Buffers and pH modifiers influence solution pH, buffer capacity, osmolality, osmolarity, and water conductivity. When used in chemical analysis, buffers must be chemically compatible with the reagents and test substance. Buffers used in physiological systems should not interfere with the pharmacological activity of the medicament or the normal function of the organism.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected pH-modifier or buffering-agent functions: *Water Conductivity* ⟨645⟩, *Osmolality and Osmolarity* ⟨785⟩, and *pH* ⟨791⟩.

Functional Category: Wetting and/or Solubilizing Agent

DESCRIPTION

Solubilizers can be used to dissolve otherwise insoluble molecules. They function by facilitating spontaneous phase transfer to yield a thermodynamically stable solution. A number of solubilizers are available commercially. Acceptable solubilizers for pharmaceutical applications have been fully evaluated in animals for safety and toxicology. Wetting agents increase the spreading and penetrating properties of a liquid by lowering its surface tension.

FUNCTIONAL MECHANISM

Wetting and solubilizing agents comprise a variety of different chemical structures or classes. Some solubilizers have unique chemical structures. For example, a hydrophilic moiety may be tethered with a hydrophobic moiety to yield distinct micelle shapes and morphologies in water, thus facilitating solubilization. The mechanism of solubilization often is associated with a favorable interaction of the insoluble agent and the interior core of the solubilizer assembly (e.g., micelles). In other cases, unique hydrophobic sites that are capable of forming inclusion complexes are present. Other types of solubilizers use a range of polymeric chains that interact with hydrophobic molecules to increase solubility by dissolving the insoluble agent into the polymeric chains.

PHYSICAL PROPERTIES

Wetting and solubilizing agents are typically solid, liquid, or waxy materials. Their physical properties depend on their chemical structures. The physical properties and performance of the wetting agents and solubilizers, however, depend on the surface-active properties of the solubilizers and on the hydrophilic–lipophilic balance (HLB). Materials with lower HLB values behave as emulsifiers, whereas those with higher HLB values typically behave as solubilizing agents. For example, the commonly

used wetting and solubilizing agent sodium lauryl sulfate (HLB 40) is hydrophilic and highly water soluble and, upon dispersion in water, spontaneously reduces surface tension and forms micelles that function to solubilize lipophilic materials.

The unique hydrophilicity and hydrophobicity properties of solubilizers can be characterized by their chemical structures, aggregate numbers, or critical micelle concentrations (CMCs). The CMC value is unique to an individual solubilizer that bears hydrophilic, lipophilic, and/or hydrophobic chains. CMC is a measure of the concentration at which the surface-active molecules aggregate. These molecular aggregates can solubilize the solute by incorporating part into the hydrophobic interior. Such interactions with the insoluble molecule further stabilize the molecules in the entire assemblies without precipitation.

CHEMICAL PROPERTIES

The chemical and surface-active properties depend on the structures of the solubilizers. Because of the complex nature of solute–solvent–solubilizer interactions, pharmaceutical scientists must carefully consider, identify, and control the CMAs of solubilizers.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected solubilizing agent functions: *Fats and Fixed Oils* ⟨401⟩, *Light Diffraction Measurement of Particle Size* ⟨429⟩, *pH* ⟨791⟩, *Specific Gravity* ⟨841⟩, *Specific Surface Area* ⟨846⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, and *Scanning Electron Microscopy* ⟨1181⟩.

General Chapters

Functional Category: Antimicrobial Preservative

DESCRIPTION

Antimicrobial preservatives are used to kill or prevent growth of bacteria, yeast, and mold in the dosage form.

FUNCTIONAL MECHANISM

Preservatives work by a variety of mechanisms to control microbes. Most work at the cell membrane, causing membrane damage and cell leakage. Other modes of action include transport inhibition, protein precipitation, and proton-conducting uncoupling. Some preservatives are bactericidal (kill bacteria or yeast and mold); some are bacteriostatic (inhibit growth of microorganisms); and others are sporicidal (kill spores). Several of the preservatives can act synergistically (e.g., combinations of parabens).

PHYSICAL PROPERTIES

Antimicrobials generally are soluble in water at concentration ranges at which they are effective. The vapor pressure of these agents is important, especially if the dosage form is intended for lyophilization or spray drying. Several of these agents are flammable. Understanding an excipient's partition coefficient is important because partitioning of a preservative into an oil phase can diminish the preservative's concentration in the aqueous phase, which in turn can reduce its value as a preservative.

CHEMICAL PROPERTIES

Phenolic preservatives can undergo oxidation and color formation. Incompatibilities of preservatives (cationic and anionic mixtures, adsorption to tubes or filters, or binding to surfactants and proteins) should be taken into account during product development.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected antimicrobial functions: *Injections and Implanted Drug Products* ⟨1⟩, *Antimicrobial Effectiveness Testing* ⟨51⟩, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Antimicrobial Agents—Content* ⟨341⟩.

ADDITIONAL INFORMATION

Safety and labeling requirements regarding the use of antimicrobial preservatives should be considered.

Functional Category: Chelating and/or Complexing Agents

DESCRIPTION

Chelating agents form soluble complex molecules with certain metal ions (e.g., copper, iron, manganese, lead, and calcium) and essentially remove the ions from solution to minimize or eliminate their ability to react with other elements and/or to precipitate. The agents are used in pharmaceuticals (oral, parenteral, and topical formulations), cosmetics, and foods to sequester ions from solution and to form stable complexes. Chelating agents also are referred to as chelants, chelators, or sequestering agents.

Complexing agents form soluble complex molecules (e.g., inclusion complexes) with other solutes (e.g., drug substances) and can influence physical and chemical properties such as solubility and stability.

FUNCTIONAL MECHANISM

Chelating agents are used to sequester undesirable metal ions from solution. The chemical structure of chelating agents allows them to act as a "claw" to associate with the metal atom by forming a heterocyclic ring structure. Complexing agents function similarly but mechanistically and do not (by definition) require a two-point claw structure because they can associate via one or more binding sites. All chelating agents are complexing agents, but not all complexing agents are chelating agents. Chelating agents are used as antioxidant synergists, antimicrobial synergists, and water softeners. By sequestering metal ions from solution, chelating agents reduce the propensity for oxidative reactions. Chelating agents also have the ability to enhance antimicrobial effectiveness by forming a metal-ion–deficient environment that otherwise could feed microbial growth.

Complexing agents generally form soluble complex molecules with solutes (e.g., drug molecules) that can influence physical, chemical, and drug delivery properties. Complexing agents that form inclusion complexes typically contain a hydrophobic cavity into which a drug substance can enter and an outer, more hydrophilic region.

PHYSICAL PROPERTIES

Chelating and complexing agents generally are soluble in water and typically are dissolved in liquid dosage forms. Physical properties may be important and should be considered because they may influence processing requirements (e.g., particle size may influence mixing time required to dissolve). Chelating and complexing agents exhibit different degrees of hygroscopicity. Because chelating agents are used in low levels, particle size distribution can be important to enable acceptable dosage form content uniformity.

CHEMICAL PROPERTIES

Chelating agents complex with metal ions via any combination of ionic and covalent bonds. Dilute aqueous solutions may be neutral, acidic, or alkaline. Edetic acid and its salts are incompatible with strong oxidizers, strong bases, and polyvalent metal ions (e.g., copper and nickel). Specific agents are selected for a formulation based on their solubility, affinity for the target metal ion, and stability. Edetate salts are more soluble than the free acid. Unlike other edetate salts and the free acid, edetate calcium disodium does not sequester calcium and therefore is preferred to prevent hypocalcemia. It is also preferred to chelate metals with the release of calcium ions. Alternatively, disodium edetate can be used to treat hypercalcemia. Edetic acid will decarboxylate if heated above 150°.

Complexing agents generally form molecular complexes with drug substances that are dependent on complexing agent physical and chemical properties. The ability of a solute to form an inclusion complex is dependent on complexing agent molecular weight, chemical structure, and the dimensions of the hydrophobic cavity.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected chelating and complexing functions: *Antimicrobial Effectiveness Testing* 〈51〉, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* 〈61〉, ● *Elemental Impurities—Limits* 〈232〉 and *Elemental Impurities—Procedures* 〈233〉, ● (Official 1-Jan-2018) *Iron* 〈241〉, *Lead* 〈251〉, *Antimicrobial Agents—Content* 〈341〉, *Light Diffraction Measurement of Particle Size* 〈429〉, *Loss on Drying* 〈731〉, *pH* 〈791〉, *Water Determination* 〈921〉, and *Cellular and Tissue-Based Products* 〈1046〉.

Functional Category: Antioxidant

DESCRIPTION

This category applies to antioxidants used as in vitro stabilizers of pharmaceutical preparations to mitigate oxidative processes. Antioxidants used for their biological activity in vivo may be regarded as active ingredients with therapeutic effects and are

not discussed. Antioxidants delay the onset of and/or significantly reduce the rate of complex oxidative reactions that could otherwise have a detrimental effect on the drug substance. Antioxidants also can be considered for protecting nonactive components such as unsaturated oils, pegylated lipids, flavors, and essential oils. Thus, antioxidants preserve the overall integrity of the dosage form against oxidative stress. Antioxidants are most effective when incorporated in the formula to prevent or delay the onset of chain reactions and to inhibit free radicals and hydroperoxides from engaging in the cascading processes described above. Effective application of antioxidants and evaluation of their efficacy necessitate an understanding of oxidative mechanisms and the nature of the byproducts they generate. Autoxidation is initiated when oxygen reacts with a substrate to form highly reactive species known as free radicals ($RH \rightarrow R\cdot$). After "initiation" the free radicals in the presence of oxygen can trigger chain reactions ($R\cdot + O_2 \rightarrow ROO\cdot$ and $ROO\cdot + RH \rightarrow R\cdot + ROOH$) to form peroxy radicals, hydroperoxides, and new alkyl radicals that can initiate and then propagate their own chain reactions. The cascading reactions during the propagation phase can be accelerated by heat, light, and metal catalysts. In the presence of trace amounts of metal catalysts (Cu^+, Cu^{2+}, Fe^{2+}, and Fe^{3+}), hydroperoxides (ROOH) readily decompose to $RO\cdot$ and $ROO\cdot$ and subsequently can trigger reactions with the API and/or the excipients (e.g., hydrocarbons) to form hydroxyl acids, keto acids, and aldehydes that can have further undesirable effects. Note that hydroperoxides are not solely the reaction products of oxidative mechanisms within a formulation. Residual amounts of hydroperoxides also can be found in commonly used excipients like polyethylene glycols, polyvinylpyrrolidone, and polysorbates. The initiation phase generally is slow and has a limited effect on the quality of the finished product. The propagation phase, in contrast, involves rapid, irreversible degradation of chemical species.

FUNCTIONAL MECHANISM

Antioxidants can be grouped by their mode of action. Phenolic antioxidants that block free radical chain reactions also are known as true or primary antioxidants. This group consists of monohydroxy or polyhydroxy phenol compounds with ring substitutions. They have very low activation energy to donate hydrogen atom(s) in exchange for the radical electrons that are rapidly delocalized by free radicals. By accepting the radical electrons, they stabilize free radicals. The reaction yields antioxidant free radicals that also can react with lipid free radicals to form other stable compounds. Thus, they can block oxidative chain reactions both in the initiation and propagation stages. Because of their solubility behavior, phenolic antioxidants are most effective in protecting oils and oil-soluble actives against oxidative stress. Reducing agents generally are water-soluble antioxidants (e.g., L-ascorbic acid) with lower redox potential than the drug or the excipient they are protecting. They delay the onset and the rate of oxidative reactions by sacrificially reacting with oxygen and other reactive species. The oxygen-scavenging potential of the reducing agents may be sensitive to pH and also can be negatively affected in the presence of trace elements. Chelating agents bind with free metals (Cu^+, Cu^{2+}, Fe^{2+}, and Fe^{3+}) that may be present in trace amounts in the formulation. The newly formed complex ions are nonreactive. Chelating agents therefore remove the capacity of the metal catalysts to participate in oxidative reactions that occur during the propagation stage.

The utility of antioxidants can be maximized by synergistic use of one or two primary antioxidants along with reducing and chelating agents. The combined effect often is greater than the sum of the individual effects of each antioxidant (synergistic effect).

PHYSICAL PROPERTIES

Solubility of the antioxidant should be greatest in the formulation phase (oily, aqueous, or emulsion interface), where the drug substance is most soluble. The temperature at which the antioxidant decomposes is critical for autoclaved preparations, where loss of antioxidant activity may occur. Stability of the antioxidant also must be considered and may be a function of pH and processing conditions. Metal ions may react with propyl gallate to form colored complexes. At alkaline pH, certain proteins and sodium salts may bring about discoloration of *tert*-butylhydroquinone.

CHEMICAL PROPERTIES

Activation energy, oxidation–reduction potential, and stability at different formulation (e.g., pH) and processing (e.g., heat) conditions are important chemical properties. If the dosage form's expected shelf life depends on the antioxidant's function, the concentration must be factored in and periodically assessed to ensure that a sufficient amount of antioxidant remains throughout the product shelf life.

GENERAL CHAPTERS

The following general chapters may be useful for assessing selected excipient antioxidant functions: *Iron* ⟨241⟩, *Chromatography* ⟨621⟩, *Crystallinity* ⟨695⟩, *Melting Range or Temperature* ⟨741⟩, *Specific Surface Area* ⟨846⟩, and *Water Determination* ⟨921⟩.

Functional Category: Sweetening Agent

DESCRIPTION

Sweetening agents are used to sweeten oral dosage forms and to mask unpleasant flavors. See *Functional Category: Flavor and Fragrance* for more details.

FUNCTIONAL METABOLISM

Sweetening agents bind to receptors on the tongue that are responsible for the sensation of sweetness. The longer the sweetener molecule remains attached to the receptor, the sweeter the substance is perceived to be. The standard for sweetness is sucrose.

PHYSICAL PROPERTIES

The primary physical properties relevant to sweeteners relate to their compatibility with the other ingredients in the formulation (e.g., acidic ingredients), processing conditions (e.g., heating), particle size and distribution, moisture content, isomerism, sweetness, and taste-masking capability. These properties may be formulation dependent.

CHEMICAL PROPERTIES

Sweeteners can be divided into three main groups: sugars (which have a ring structure), sugar alcohols (sugars that do not have a ring structure), and artificial sweeteners. All sweeteners are water soluble. The stability of many sweeteners is affected by pH and other ingredients in the formulation. Some sweeteners may catalyze the degradation of some active ingredients, especially in liquids and in cases in which the manufacturing processes involve heating.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected sweetening functions: *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Loss on Drying* ⟨731⟩, *Melting Range or Temperature* ⟨741⟩, *Optical Rotation* ⟨781⟩, and *Water Determination* ⟨921⟩.

ADDITIONAL INFORMATION

Products that contain aspartame must include a warning on the label stating that the product contains phenylalanine. Sugar alcohols have a glycemic index well below that of glucose. However, sorbitol is slowly metabolized to fructose and glucose, which raises blood sugar levels. Sugar alcohols in quantities generally greater than 20 g/day act as an osmotic laxative, especially when they are contained in a liquid formulation. Preservative systems should be carefully chosen to avoid incompatibility with the sweetener, and some sweeteners are incompatible with certain preservatives.

SEMISOLIDS, TOPICALS, AND SUPPOSITORIES

Functional Category: Suppository Base

DESCRIPTION

Suppository bases are used in the manufacture of suppositories (for rectal administration) and pessaries (for vaginal administration). They can be hydrophobic or hydrophilic.

FUNCTIONAL MECHANISM

Suppositories should melt at just below body temperature (37°), thereby allowing the drug to be released either by erosion and partition if the drug is dissolved in the base or by erosion and dissolution if the drug is suspended in the base. Hard fat suppository bases melt at approximately body temperature. Hydrophilic suppository bases also melt at body temperature and typically also dissolve or disperse in aqueous media. Thus, release takes place via a combination of erosion and dissolution.

PHYSICAL PROPERTIES

The important physical characteristic of suppository bases is melting range. In general, suppository bases melt between 27° and 45°. However, individual bases usually have a much narrower melting range within these temperature boundaries, typically 2°–3°. The choice of a particular melting range is dictated by the influence of the other formulation components on the melting range of the final product.

CHEMICAL PROPERTIES

Hard fat suppository bases are mixtures of semisynthetic triglyceride esters of longer-chain fatty acids. They may contain varying proportions of mono- and diglycerides and also may contain ethoxylated fatty acids. They are available in many different grades that are differentiated by melting range, hydroxyl number, acid value, iodine value, solidification range, and saponification number.

Hydrophilic suppository bases are mixtures of hydrophilic semisolid materials that in combination are solid at room temperature and yet release the drug by melting, erosion, and dissolution when administered to the patient. Hydrophilic suppository bases have much higher levels of hydroxyl groups or other hydrophilic groups than do hard fat suppository bases. Polyethylene glycols that show appropriate melting behavior are examples of hydrophilic suppository bases.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected suppository base functions: *Fats and Fixed Oils* 〈401〉, *Congealing Temperature* 〈651〉, *Melting Range or Temperature* 〈741〉, and *Pharmaceutical Dosage Forms* 〈1151〉.

ADDITIONAL INFORMATION

Some materials included in suppositories based on hard fats have much higher melting ranges. These materials typically are microcrystalline waxes that help stabilize molten suspension formulations. Suppositories also can be manufactured from glycerinated gelatin.

Functional Category: Suspending and/or Viscosity-Increasing Agents

DESCRIPTION

Suspending and viscosity-increasing agents are used in pharmaceutical formulations to stabilize dispersal systems (e.g., suspensions or emulsions), to reduce the rate of solute or particulate transport, or to decrease the fluidity of liquid formulations.

FUNCTIONAL MECHANISMS

A number of mechanisms contribute to the dispersion stabilization or viscosity-increasing effect of these agents. The most common is the increase in viscosity—because of the entrapment of solvent by macromolecular chains or clay platelets—and the disruption of laminar flow. Other mechanisms include gel formation via a three-dimensional network of excipient molecules or particles throughout the solvent continuum and steric stabilization wherein the macromolecular or mineral component in the dispersion medium adsorbs to the surfaces of particles or droplets of the dispersed phase. The latter two mechanisms increase formulation stability by immobilizing the dispersed phase.

PHYSICAL PROPERTIES

Each of the mechanisms—increased viscosity, gel formation, or steric stabilization—is a manifestation of the rheological character of the excipient. Because of the molecular weights and sizes of these excipients, the rheological profiles of their dispersions are non-Newtonian. Dispersions of these excipients display viscoelastic properties. The molecular weight distribution and polydispersity of the macromolecular excipients in this category are important criteria for their characterization.

CHEMICAL PROPERTIES

The majority of the suspending and viscosity-increasing agents are: 1) hydrophilic carbohydrate macromolecules (acacia, agar, alginic acid, carboxymethylcellulose, carrageenans, dextrin, gellan gum, guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hypromellose, maltodextrin, methylcellulose, pectin, propylene glycol alginate, sodium alginate, starch, tragacanth, and xanthan gum); and 2) noncarbohydrate hydrophilic macromolecules, including gelatin, povidone carbomers, polyethylene oxide, and polyvinyl alcohol. Minerals (e.g., attapulgite, bentonite, magnesium aluminum silicate, and silicon dioxide) comprise the second-largest group of suspending and viscosity-increasing agents. Aluminum monostearate is the one

non-macromolecular, nonmineral excipient in this functional category. It consists chiefly of variable proportions of aluminum monostearate and aluminum monopalmitate.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected viscosity-increasing functions: *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

Functional Category: Ointment Base

DESCRIPTION

An ointment is a viscous semisolid preparation used topically on a variety of body surfaces. An ointment base is the major component of an ointment and controls its physical properties.

FUNCTIONAL MECHANISM

Ointment bases serve as vehicles for topical application of medicinal substances and also as emollients and protective agents for skin.

PHYSICAL PROPERTIES

Ointment bases are liquids with a relatively high viscosity so that solids can be suspended as a stable mixture.

Ointment bases are classified as: 1) oleaginous ointment bases that are anhydrous, do not absorb water readily, are insoluble in water, and are not removable by water (e.g., petrolatum); 2) absorption ointment bases that are anhydrous and absorb some water but are insoluble in water and are not water removable (e.g., lanolin); 3) emulsion ointment bases that are water-in-oil or oil-in-water emulsions and are hydrous, absorb water, and are insoluble in water (e.g., creams of water, oils, waxes, or paraffins); and 4) water-soluble ointment bases that are anhydrous and absorb water and are soluble in water and are water removable (e.g., polyethylene glycol).

CHEMICAL PROPERTIES

Ointment bases are selected to be inert and chemically stable.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected ointment base functions: *Congealing Temperature* ⟨651⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

Functional Category: Stiffening Agent

DESCRIPTION

A stiffening agent is an agent or a mixture of agents that increases the viscosity or hardness of a preparation, especially in ointments and creams.

FUNCTIONAL MECHANISM

In general, stiffening agents are high melting point solids that increase the melting point of ointments or increase the consistency or body of creams. Stiffening agents can be either hydrophobic (e.g., hard fat or paraffin) or hydrophilic (e.g., polyethylene glycol, high molecular weight).

PHYSICAL PROPERTIES

The primary physical property relevant to stiffening agents is their high melting point or melting range. Typical melting ranges for stiffening agents range from 43° to 47° (cetyl esters wax), 53° to 57° (glyceryl distearate), 69° to 74° (glyceryl behenate), and 85° to 88° (castor oil, hydrogenated).

CHEMICAL PROPERTIES

Stiffening agents comprise a diverse group of materials that include glycerides of saturated fatty acids, solid aliphatic alcohols, esters of saturated fatty alcohols and saturated fatty acids, saturated hydrocarbons, blends of fatty alcohols and a polyoxyethylene derivative of a fatty acid ester of sorbitan, and higher ethylene glycol polymers.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected stiffening-agent functions: *Congealing Temperature* ⟨651⟩, *Melting Range or Temperature* ⟨741⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

ADDITIONAL INFORMATION

Some of the materials included as stiffening agents increase the water-holding capacity of ointments (e.g., petrolatum) or function as co-emulsifiers in creams. Examples include stearyl alcohol and cetyl alcohol.

Functional Category: Emollient

DESCRIPTION

Emollients are excipients used in topical preparations to impart lubrication, spreading ease, texture, and softening of the skin and to counter the potentially drying/irritating effect of surfactants on the skin.

FUNCTIONAL MECHANISM

Emollients help form a protective film and maintain the barrier function of the epidermis. Their efficacy may be described by three mechanisms of action: protection against the delipidizing and drying effects of surfactants, humectancy due to occlusion (by providing a layer of oil on the surface of the skin, emollients slow water loss and thus increase the moisture-retention capacity of the stratum corneum), and lubricity, adding slip or glide to the preparation.

PHYSICAL PROPERTIES

Emollients impart one or more of the following attributes to a pharmaceutical preparation: spreading capacity, pleasant feel to the touch, softness of the skin, and indirect moisturization of the skin by preventing transepidermal water loss.

CHEMICAL PROPERTIES

Emollients are either oils or are derived from components of oils as esters of fatty acids. Depending on the nature of its fatty acid ester, an emollient may be liquid, semisolid, or solid at room temperature. Generally, the higher the molecular weight of the fatty acid moiety (carbon chain length) the richer the feel and softness of the touch. Fluidity generally is imparted by shorter chain length and higher degree of unsaturation in the fatty acid moiety. The degree of branching of ester bonds also influences the emollient properties.

GENERAL CHAPTER

The following general chapter may be useful in ensuring consistency in selected emollient functions: *Fats and Fixed Oils* ⟨401⟩.

PARENTERALS

Functional Category: Pharmaceutical Water

DESCRIPTION

Water is used as a solvent, vehicle, diluent, or filler for many drug products, especially those supplied in liquid form. These may include injectable drugs, ophthalmic drugs, inhalation solutions, and others. Water also is a vehicle for buffers and antimicrobial agents and is a volume expander for infusion solutions.

USP includes monographs for eight grades of pharmaceutical waters. Water for Injection is intended for use in the preparation of parenteral solutions. Where used for the preparation of parenteral solutions subject to final sterilization, use suitable means to minimize microbial growth, or first render the Sterile Water for Injection and, thereafter, protect it from microbial contamination. For parenteral solutions that are prepared under aseptic conditions and are not sterilized by appropriate filtration or in the final container, first render the Sterile Water for Injection and, thereafter, protect it from microbial contamination. Do not use Purified Water in preparations intended for parenteral administration. Where used for sterile dosage forms other than for parenteral administration, process the article to meet the requirements under *Sterility Tests* 〈71〉, or first render the Sterile Purified Water and, thereafter, protect it from microbial contamination. *USP* also contains references to other types of water, such as distilled water, deionized water, and others according to specific use as summarized in general information chapter *Water for Pharmaceutical Purposes* 〈1231〉.

FUNCTIONAL MECHANISM

A solvent is able to dissolve materials because it is able to disrupt the intermolecular attractive forces and to allow the individual molecules to become dispersed throughout the bulk solvent. Water is a favored solvent and vehicle in the majority of applications because it is easy to handle, safe, and inexpensive.

PHYSICAL PROPERTIES

Water is liquid at normal temperature and pressure. It forms ice at the freezing temperatures of 0° or lower, and it vaporizes at a normal boiling temperature of 100°, depending on atmospheric pressure. Vaporized water in the form of steam is used for sterilization purposes because the latent heat of steam is significantly higher than that of boiling water.

CHEMICAL PROPERTIES

Water in its pure form is neutral in pH and has very low conductivity and total organic carbon (TOC). However, pH, conductivity, and TOC are affected by storage conditions and exposure to gases in the air. Exposure to atmospheric carbon dioxide lowers water's pH. Storage in plastic containers may increase the TOC content of water over time. Water stored in glass containers may result in an increase in pH and conductivity over time.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected pharmaceutical water functions: *Injections and Implanted Drug Products* 〈1〉, *Bacterial Endotoxins Test* 〈85〉, *Total Organic Carbon* 〈643〉, *Water Conductivity* 〈645〉, *Water for Hemodialysis Applications* 〈1230〉, and *Water for Pharmaceutical Purposes* 〈1231〉.

Functional Category: Bulking Agent

DESCRIPTION

Bulking agents used in lyophilized pharmaceuticals, also referred to as freeze-dried products, include various saccharides, sugar alcohols, amino acids, and polymers. The primary function of bulking agents is to provide a pharmaceutically elegant freeze-dried cake with noncollapsible structural integrity that will reconstitute rapidly before administration. In addition, bulking agents are selected to prevent product loss caused by blow-out during freeze drying, to facilitate efficient drying, and to provide a physically and chemically stable formulation matrix. Complementary combinations of bulking agents, e.g., mannitol and a polymer, frequently are used to improve performance.

FUNCTIONAL MECHANISM

A bulking agent that readily crystallizes during lyophilization helps maintain the structural integrity of the cake formed during primary drying, thereby preventing macroscopic collapse and maintaining pharmaceutical elegance. Microscopic collapse of amorphous components in the formulation can still occur (with some potentially undesirable results) but does not result in macroscopic collapse or "meltback" if the bulking agent's properties and concentration are adequate. The bulking agent also should possess a high eutectic melting temperature with ice to permit relatively high primary drying temperatures with commensurate rapid and efficient drying and subsequent rapid reconstitution upon usage. Functional cake-forming excipients, such as mannitol, frequently are used because they crystallize during freezing, thereby allowing efficient drying and the formation of a structurally robust and stable cake. Amino acids and cosolvents also have been used to achieve this effect. Most biopolymer active ingredients remain amorphous upon freeze-drying, and bulking agents such as disaccharides can function as lyoprotectants by helping to maintain a stable amorphous phase during freezing and drying to prevent denaturation. Solubility enhancement of an insoluble crystalline active ingredient sometimes is achieved with the use of a biopolymer that enhances

solubility or prevents crystallization during lyophilization or subsequent reconstitution. Bulking agents also are selected on the basis of biocompatibility, buffering capability, and tonicity-modifying properties.

Lyoprotectant properties of bulking agents (i.e., those that protect the drug substance during lyophilization) typically are achieved by the formation of a highly viscous glassy phase that includes the biopolymer drug substance in combination with low molecular weight amorphous saccharides such as sucrose, trehalose, or certain amino acids. A typical approach for protein pharmaceutical formulation is to combine a sugar alcohol that readily crystallizes and an amorphous diluent. This mixture acts as a lyoprotectant.

PHYSICAL PROPERTIES

Bulking agents are dissolved in aqueous solution before lyophilization. Therefore, chemical purity and the absence of bioburden and pyrogenic materials are essential properties of the excipient. However, the physical form and particle properties of the excipient generally are not relevant to the final properties of the lyophilized formulation. The solubilization process and the drying process can be facilitated by the use of volatile cosolvents such as ethanol or tertiary butyl alcohol.

The physical properties that are essential to product performance during and after lyophilization include the glass transition temperature (T_g) of the amorphous frozen concentrate before drying, the glass transition temperature of the final dried formulation cake, and the eutectic melting temperature of the crystalline bulking agent with ice. The glass transition temperature (T_g) of the formulation depends on the glass transition temperatures of the individual components, concentrations, and interactions. Although approximations can be made based on reported transition temperatures for individual components, current practice includes the measurement of formulation glass transition temperatures by thermal analysis or freeze-drying microscopy.

The physical states of the bulking agent during and after lyophilization are important physical properties. Both formulation composition and processing parameters play roles in determining whether the bulking agent is amorphous or takes a specific crystalline form. Rate of freezing, drying temperatures, and annealing are among the important process parameters used to control the physical state of the formulation and its components. Moisture retention and adsorption after lyophilization also can contribute to formulation instability and poor reconstitution.

CHEMICAL PROPERTIES

Reactivity of the bulking agent with other formulation components, especially the active ingredient, may be critical. Reducing sugars are well known to react with aromatic and aliphatic amines. Glycols may contain trace peroxide levels that can initiate oxidative degradation. The ability of saccharides and polyhydric alcohols to form hydrogen bonds to biopolymers may play a role in their lyoprotection effects.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selecting bulking agent functions: *Injections and Implanted Drug Products* ⟨1⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Thermal Analysis* ⟨891⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, and *Water–Solid Interactions in Pharmaceutical Systems* ⟨1241⟩.

Functional Category: Tonicity Agent

DESCRIPTION

To avoid crenation or hemolysis of red blood cells and to mitigate pain and discomfort if solutions are injected or introduced into the eyes and nose, solutions should be made isotonic. This requires that the effective osmotic pressure of solutions for injection must be approximately the same as that of blood. When drug products are prepared for administration to membranes, such as eyes or nasal or vaginal tissues, solutions should be made isotonic with respect to these tissues.

FUNCTIONAL MECHANISM

Tonicity is equal to the sum of the concentrations of the solutes that have the capacity to exert an osmotic force across a membrane and thus reflects overall osmolality. Tonicity applies to the impermeant solutes within a solvent—in contrast to osmolarity, which takes into account both permeant and impermeant solutes. For example, urea is a permeant solute, meaning that it can pass through the cell membrane freely and is not factored when determining the tonicity of a solution. In contrast, sodium chloride is impermeant and cannot pass through a membrane without the help of a concentration gradient and, therefore, contributes to a solution's tonicity.

PHYSICAL PROPERTIES

Solutions of sodium chloride, dextrose, and Lactated Ringer's are common examples of pharmaceutical preparations that contain tonicity agents. Not all solutes contribute to the tonicity, which in general depends only on the number of solute particles present in a solution, not the kinds of solute particles. For example, mole for mole, sodium chloride solutions display a higher osmotic pressure than glucose solutions of the same molar concentration. This is because when glucose dissolves, it remains one particle, but when sodium chloride dissolves, it becomes two particles: Na^+ and Cl^-. Several methods are available to calculate tonicity.

CHEMICAL PROPERTIES

Tonicity agents may be present as ionic or nonionic types. Examples of ionic tonicity agents are alkali metal or earth metal halides such as calcium chloride ($CaCl_2$), potassium bromide (KBr), potassium chloride (KCl), lithium chloride (LiCl), sodium iodide (NaI), sodium bromide (NaBr) or sodium chloride (NaCl), sodium sulfate (Na_2SO_4), or boric acid. Nonionic tonicity agents include glycerol, sorbitol, mannitol, propylene glycol, or dextrose. Sodium or potassium chloride and dextrose commonly are added to adjust tonicity.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected tonicity agent functions: *Injections and Implanted Drug Products* ⟨1⟩, *Osmolality and Osmolarity* ⟨785⟩, and *Pharmaceutical Calculations in Pharmacy Practice* ⟨1160⟩.

AEROSOLS

Functional Category: Propellant

DESCRIPTION

Propellants are compounds that are gaseous under ambient conditions. They are used in pharmaceuticals (nasal sprays and respiratory and topical formulations), cosmetics, and foods to provide force to expel contents from a container.

FUNCTIONAL MECHANISM

Propellant substances are low boiling point liquids or compressed gases that are relatively inert toward active ingredients and excipients. They can be characterized by three properties: whether they form a liquid phase at ambient temperatures and useful pressures, their solubility and/or miscibility in the rest of the formulation, and their flammability. Their performance is judged by their ability to provide adequate and predictable pressure throughout the usage life of the product.

Propellants that have both a liquid and gas phase in the product provide consistent pressures as long as there is a liquid phase present—the pressure in the headspace is maintained by the equilibrium between the two phases. In contrast, the pressure provided by propellants that have no liquid phase may change relatively rapidly as the contents of the container are expelled. As the headspace becomes larger, the pressure within the container falls proportionately. Propellants that have no liquid phase but have significant pressure-dependent solubility in the rest of the formulation have performance characteristics between those of the other two systems. In such cases, as the headspace increases the propellant comes out of solution to help to maintain the pressure of the system.

In metered-dose inhalers, the propellant has a liquid phase that is an integral part of the dispensed pharmaceutical product. Actuating the metering valve dispenses a defined volume of the liquid contents. The propellant spontaneously boils and provides atomizing and propulsive force. A predictable change in active concentration occurs from the beginning to the end of the container life cycle as the liquid phase of the propellant vaporizes to reestablish the equilibrium pressure of the system as the headspace increases.

PHYSICAL PROPERTIES

Propellants have boiling points well below ambient temperatures. A propellant's density for disperse systems and its solubility properties may be significant considerations when one selects a propellant. Apaflurane and norflurane have liquid-phase densities that are greater than that of water. Hydrocarbon propellants (butane, isobutane, and propane) and dimethyl ether have liquid-phase densities that are less than that of water.

CHEMICAL PROPERTIES

Propellants typically are stable materials. The hydrocarbon propellants (butane, isobutene, and propane) and dimethyl ether are all flammable materials. Apaflurane, carbon dioxide, nitrogen, and norflurane are nonflammable. Nitrous oxide is not flammable but supports combustion. Chlorofluorocarbon propellants are considered to be ozone-depleting substances. Their use in foods, drugs, devices, or cosmetics is regulated by 21 CFR 2.125. Albuterol metered-dose inhalers formulated with chlorofluorocarbon propellants have not been available in the United States since January 1, 2009.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected propellant functions: *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Chromatography* ⟨621⟩, and *Water Determination* ⟨921⟩.

Change to read:

DRY POWDER INHALERS

Dry powder inhalers (DPIs) commonly contain few functional excipients. For example, DPIs may contain a carrier and may use a capsule shell. Other useful excipients include glidants to improve flow during manufacture of the active carrier mix. A discussion of the use of a lubricant can be found in the tablet or capsule sections above in addition to the carrier properties discussed below.

Functional Category: Carrier

DESCRIPTION

Carriers are used to help deposit the active ingredient in the lung and may have a secondary role in diluting the active to ensure that dosages can be properly metered.

FUNCTIONAL MECHANISM

The carriers are used to promote drug deposition into the lungs for better penetration or absorption in the appropriate lung location. In addition, the carrier is used to decrease the concentration of the active so the latter is adequately dosed in a uniform manner.

PHYSICAL PROPERTIES

The physical properties of carriers include appropriate morphology, hydration state, flowability, surface energy, and particle size distribution.

CHEMICAL PROPERTIES

Carriers must have suitable purity, including low microbial content and no extraneous proteins or impurities, to avoid interactions with the patient's immune system.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected carrier functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, ●*Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩,● (Official 1-Jan-2018) *Light Diffraction Measurement of Particle Size* ⟨429⟩, *Nitrogen Determination* ⟨461⟩, *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Crystallinity* ⟨695⟩, *Characterization of Crystalline Solids by Microcalorimetry and Solution Calorimetry* ⟨696⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Powder Fineness* ⟨811⟩, *Mid-Infrared Spectroscopy* ⟨854⟩ and *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *Water Determination* ⟨921⟩, *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* ⟨941⟩, and *Powder Flow* ⟨1174⟩.

Functional Category: DPI Capsule Shells

DESCRIPTION

Capsule shells sometimes are used in DPIs. The capsule shell is used to contain the dosage amount and safeguard the inhalable powder in a DPI.

FUNCTIONAL MECHANISM

The use of capsule shell may speed pharmaceutical development because it does not require a complex device and can use premeasured drug substance or formulation. A capsule shell must not fragment into inhalable portions and should remain intact after the shell breaks to expose the powder for inhalation.

PHYSICAL PROPERTIES

Capsule shell composition generally is dictated by the drug substance's moisture content, brittleness, and electrostatic interactions with the inhalable powder.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected DPI capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, *Arsenic* ⟨211⟩, ●*Elemental Impurities—Limits* ⟨232⟩ and *Elemental Impurities—Procedures* ⟨233⟩, ● (Official 1-Jan-2018) *Residue on Ignition* ⟨281⟩, *Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩, *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Loss on Drying* ⟨731⟩, *Optical Microscopy* ⟨776⟩, *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, *Uniformity of Dosage Units* ⟨905⟩, *Water Determination* ⟨921⟩, *Color—Instrumental Measurement* ⟨1061⟩, and *Water–Solid Interactions in Pharmaceutical Systems* ⟨1241⟩.

ADDITIONAL INFORMATION

In addition to the general chapters listed above, useful information for ensuring consistency in selected capsule shell functions may be found in *Gelatin, Gel Strength (Bloom Value)*.

OPHTHALMIC PREPARATIONS

Functional Category: Antimicrobial Preservatives

DESCRIPTION

The preservative system acts as a safeguard to kill or inhibit the growth of microorganisms that may be inadvertently introduced in the product after the manufacturing process either during storage or use.

FUNCTIONAL MECHANISM

Antimicrobial preservatives work by a number of mechanisms. Quaternary ammonium compounds affect microbial cell membranes via charge interactions with phospholipids, leading to disruption of the cell membrane. Parabens also disrupt cell membrane integrity. Alcohols such as chlorbutanol and benzyl alcohol work via lipid (membrane) solvation and protein denaturation. *N*-[3-(Dimethylamino)propyl]tetradecanamide has greater antimicrobial effectiveness toward fungi and protozoa than do quaternary ammonium compounds. Similar to quaternary ammonium compounds, it disrupts plasma membrane integrity. Sorbic acid works by reduction of the sulfhydryl groups of proteins. Hypochlorite is a strong oxidizing agent. Reactions of chloramines with the amine groups of proteins can cause changes in conformation and thus loss of protein activity. Chlorine released by these reactions can react with cellular constituents, such as proteins and lipid. Polyaminopropyl biguanide accumulates in the cell membrane, blocking the entry of nutrients.

PHYSICAL PROPERTIES

To serve as an ophthalmic antimicrobial preservative, a compound should be at least sparingly soluble in water, thus providing an appreciable range of usable concentrations.

General Chapters

CHEMICAL PROPERTIES

A preservative must be compatible with the active and inactive ingredients of the finished product. For example, quaternary ammonium compounds are incompatible with anionic surfactants. Benzyl alcohol is incompatible with oxidizing agents. Chlorbutanol is incompatible with some nonionic surfactants. Compatibility between compounds varies with the pH of the formula. The preservative should be stable in solution at the formulation pH, usually from 5 to 8. Formulation pH can affect preservative activity by influencing how the preservative partitions between the formulation and microbes and how the preservative interacts with the target sites of the microbial cell. For example, preservatives that must pass through cell membranes before exerting activity should be formulated at a pH at which the preservative is mainly in its un-ionized state.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistent functions of selected antimicrobial preservatives: *Antimicrobial Effectiveness Testing* ⟨51⟩, *Sterility Tests* ⟨71⟩, *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, *Powder Flow* ⟨1174⟩, *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩, and *Validation of Microbial Recovery from Pharmacopeial Articles* ⟨1227⟩.

Functional Category: Polymers for Ophthalmic Use

DESCRIPTION

Polymers are used in ophthalmic preparations to enhance the retention of active ingredients by reducing the amount of product that is lost from the eye when the patient blinks. In addition, polymers also can be components of artificial tears. Most water-soluble polymers commonly used as film-forming agents in ophthalmic preparations can be categorized as follows: 1) cellulose-based substances, 2) biologically produced gums, and 3) synthetically produced substances.

FUNCTIONAL MECHANISM

Film-forming agents for ophthalmic preparations can enhance the retention of active ingredients in the eye by a number of mechanisms. They can be used as simple viscosity-modifying agents to reduce the flow of the product, thereby slowing the rate of product loss after administration. They also can be used to form films on the surface of the eye so the drug remains deposited on the eye after the liquid portion of the product has been expelled or has evaporated. These agents can be formulated to produce a film or a gel when the product warms to body temperature (upon contacting the surface of the eye), mixing with the tear film, and/or evaporating. Some polymers have shown bio-adhesive properties on the cornea and can increase drug retention.

PHYSICAL PROPERTIES

To serve as an ophthalmic film-forming agent, a polymer typically must be at least slightly soluble in water, thus providing an appreciable range of usable concentrations. Such polymers often increase viscosity or exhibit film- or gel-forming properties when warmed to body temperature, when exposed to the pH or solute composition and ionic strength of the tear film, or when the product evaporates.

CHEMICAL PROPERTIES

The finished product viscosity range that can be obtained with a film-forming agent is related to its chemical structure and molecular weight. Functional groups such as the pyruvate and acetate groups of xanthan gum can affect the relationship between viscosity and solution pH and ionic strength and also can determine film- and gel-forming properties. Polymer charge can influence interactions with the mucous layer of the eye. Molecular conformation, chain mobility, and degree of cross-linking also can affect the degree of swelling and thus performance.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistent functions of polymers for ophthalmic use: *Bulk Density and Tapped Density of Powders* ⟨616⟩, *Chromatography* ⟨621⟩, *Density of Solids* ⟨699⟩, *Loss on Drying* ⟨731⟩, *Particulate Matter in Ophthalmic Solutions* ⟨789⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, *Viscosity—Rolling Ball Method* ⟨913⟩, *Pharmaceutical Dosage Forms* ⟨1151⟩, and *Powder Flow* ⟨1174⟩. In addition, the general chapters listed under *Functional Category: Film-Forming Agents* also may be appropriate for the evaluation of polymers for ophthalmic use.

TRANSDERMALS AND PATCHES

Functional Category: Adhesive

DESCRIPTION

Topical drug delivery systems (e.g., transdermals or skin patches) require the use of adhesives to maintain contact between the applied drug delivery system and the skin. Adhesives can be intercalated as a separate layer between the formulation matrix and the skin surface, incorporated as a part of the formulation matrix itself, or applied to the periphery of the topical delivery system.

FUNCTIONAL MECHANISM

Adhesion is the tendency of dissimilar surfaces to adhere to one another as a result of one or more types of interactions. For topical drug delivery systems, these adhesive interactions generally are chemical (primarily electrostatic) or dispersive (van der Waals and/or hydrogen bonding) in nature, although there is the possibility of mechanical interaction via the interlocking of microscopic asperities.

PHYSICAL PROPERTIES

In general, the adhesives used in transdermals or skin patches are pressure-sensitive materials whose performance is best characterized by physical test methods for tackiness and viscoelasticity of the adhesive per se and viscosity of a solution of the adhesive.

CHEMICAL PROPERTIES

In transdermals, the most widely used pressure-sensitive adhesives are acrylic, rubber, and silicone polymers. Acrylic polymer adhesives include various esters of acrylic or methacrylic acid, acrylamide, methacrylamide, *N*-alkoxyalkyl, or *N*-alkyl-acrylamides. Polyisobutylenes and polysiloxanes are among the most common rubber-based and silicone-based adhesives, respectively. The molecular weight and compositional distribution of the polymers are critical to the replication of the adhesive's efficacy from batch to batch.

GENERAL CHAPTERS

The following general chapters may be useful in evaluating the suitability of adhesives used in transdermals: *Tensile Strength* 〈881〉 and *Viscosity—Capillary Methods* 〈911〉.

Functional Category: Film-Forming Agent

DESCRIPTION

Film-forming agents used as the formulation matrix of topical drug delivery systems (e.g., transdermals or skin patches) or in conjunction with such systems comprise a flexible, nontacky but adherent film, in whole or in part, applied to the skin surface.

FUNCTIONAL MECHANISM

Film formation results from the progressive loss of solvent (or dispersion medium) from a solution (or dispersion) of a film-forming agent, whether in particulate or molecularly dispersed form. Solvent (or dispersion medium) loss leads to closer molecular or particulate packing and increased interaction among the film-forming agent molecules or particles. Ultimately, a continuous film is formed as a result of increased molecular entanglement or particulate sintering.

PHYSICAL PROPERTIES

Properties critical to successful film formation include the film-forming agent's glass transition temperature (T_g), the viscosity of the solution or dispersion, and the surface characteristics of the substrate. Viscoelastic properties such as elastic modulus, viscous modulus, and intrinsic or complex viscosity describe functional characteristics, such as adhesion, for a pressure-sensitive adhesive component. Adhesion to a substrate and tack and shear tests can be used for batch release.

General Chapters

CHEMICAL PROPERTIES

Typical film-forming agents are thermoplastic or thermosetting high molecular weight polymers or copolymers, often in the form of aqueous dispersions or latex compositions. Cellulosic polymers, vinyl polymers and copolymers, and acrylic and methacrylic acid polymers and copolymers frequently are used in topical delivery systems as film-forming agents.

GENERAL CHAPTERS

The following general chapters may be useful in evaluating the suitability of film-forming agents used in transdermals and patches: *Thermal Analysis* ⟨891⟩, *Viscosity—Capillary Methods* ⟨911⟩, *Viscosity—Rotational Methods* ⟨912⟩, and *Viscosity—Rolling Ball Method* ⟨913⟩.

RADIOPHARMACEUTICALS

Radiopharmaceuticals commonly contain categories of excipients that also are used in conventional drugs. For example, radiopharmaceutical capsules may contain diluents and necessarily use a capsule shell, and parenteral radiopharmaceuticals may contain pharmaceutical water, diluents, tonicity agents, pH modifiers, antimicrobial preservatives, chelating and/or complexing agents, and antioxidants. Many radiopharmaceuticals differ from conventional drugs, however, because their preparation (reconstitution) involves one or more chemical reactions that require unusual excipients. Furthermore, the self-absorption of emitted radiation may result in the radiolytic decomposition of many radiopharmaceuticals. Hence, several excipients are used predominately in radiopharmaceutical formulations, although they occasionally may be used for other drugs.

General Chapters

Functional Category: Reducing Agent

DESCRIPTION

Reducing agents generally are required for technetium Tc 99m radiopharmaceuticals. Technetium Tc 99m, in the chemical form of sodium pertechnetate (+7 oxidation state), must be reduced to a lower oxidation state so that it can be chelated or otherwise complexed by the intended ligand to form the final Tc 99m radiopharmaceutical. The reducing agent, typically a stannous salt, generally is formulated in the kit for the preparation of the technetium Tc 99m radiopharmaceutical.

FUNCTIONAL MECHANISM

The reducing agent (e.g., stannous ion) must be present in sufficient quantity to reduce all of the technetium atoms to the intended oxidation state but must not produce undesired reduction products or other impurities (e.g., stannous hydroxide precipitates).

PHYSICAL PROPERTIES

Reducing agents (e.g., stannous salts) must be readily soluble in water.

CHEMICAL PROPERTIES

Reducing agents (e.g., stannous salts) are sensitive to oxidation by atmospheric oxygen and oxidizing species in solution. Hence, lyophilized contents of kit vials must be filled with a nonoxidative gas such as nitrogen or argon. The reducing agent also must be stable at the intended pH of the formulated product.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected reducing agent functions: *Chromatography* ⟨621⟩ and *Radioactivity* ⟨821⟩.

Functional Category: Transfer Ligand

DESCRIPTION

In the preparation of certain radiopharmaceuticals, the radiometal (e.g., stannous-reduced technetium Tc 99m) is first chelated by a relatively weak chelating ligand and then is transferred to the principal chelating ligand or complexing moiety. Examples of such transfer ligands include citrate, gluconate, and tartrate.

FUNCTIONAL MECHANISM

Transfer ligands typically undergo rapid reactions with reduced technetium to form weak chelates, thus keeping the reduced technetium in a soluble form until it is transferred to the principal ligand. This procedure is especially useful when the kinetics of complexation with the principal ligand is slow or when a heating step is necessary to expose chelating groups on the principal ligand.

PHYSICAL PROPERTIES

Transfer ligands must be readily soluble in water.

CHEMICAL PROPERTIES

Transfer ligands must have rapid complexation kinetics and must form relatively weak chelates compared to complexation with the principal ligand.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected transfer ligand functions: *Chromatography* 〈621〉 and *Radioactivity* 〈821〉.

Functional Category: Colloid Stabilizing Agent

DESCRIPTION

Lyophobic colloids tend to clump together and form large aggregates to minimize their surface-area-to-volume ratio. Colloid stabilizing agents are relatively large lyophilic molecules that coat the surface of each individual colloid particle and prevent or inhibit clumping. Examples of colloid stabilizing agents include gelatin and dextran.

FUNCTIONAL MECHANISM

The colloid stabilizing agent coats the surface of the lyophobic colloid particles, making them appear lyophilic. Additionally, the colloid stabilizing agent may be charged, thus causing the coated colloid particles to repel one another.

PHYSICAL PROPERTIES

Colloid stabilizing agents must be readily soluble in water.

CHEMICAL PROPERTIES

Colloid stabilizing agents must be capable of coating the lyophobic colloid particles, e.g., by electrostatic attraction of an opposite charge.

GENERAL CHAPTERS

The following general chapters may be useful in ensuring consistency in selected colloid stabilizing agent functions: *Chromatography* 〈621〉 and *Radioactivity* 〈821〉.

Functional Category: Free Radical Scavenger

DESCRIPTION

Radiation interactions with water and other molecules frequently produce free radicals. Free radical scavengers preferentially interact with oxidative or reductive free radicals that otherwise would result in degradation of formulation components. In the case of radiopharmaceuticals, free radical scavengers can be used to enhance radiochemical purity. Examples of free radical scavengers include methylene blue and aminobenzoic acid.

FUNCTIONAL MECHANISM

Free radical scavengers preferentially interact with radiolytically produced free radicals before these free radicals can interact with the radiopharmaceutical and produce radiochemical impurities.

PHYSICAL PROPERTIES

Free radical scavengers must be readily soluble in water.

CHEMICAL PROPERTIES

Free radical scavengers must be capable of preferentially interacting with free radicals without causing other effects.

⟨1061⟩ COLOR—INSTRUMENTAL MEASUREMENT

The observed color (see ⟨631⟩ *Color and Achromicity*) of an object depends on the spectral energy of the illumination, the absorbing characteristics of the object, and the visual sensitivity of the observer over the visible range. Similarly, it is essential that any instrumental method that is widely applicable take these same factors into account.

Instrumental methods for measurement of color provide more objective data than the subjective viewing of colors by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate and precise measurements of color and color differences that do not drift with time. The basis of any instrumental measurement of color is that the human eye has been shown to detect color via three "receptors." Hence, all colors can be broken down into a mixture of three radiant stimuli that are suitably chosen to excite all three receptors in the eye. Although no single set of real light sources can be used to match all colors (i.e., for any three lights chosen, some colors require a negative amount of one or more of the lights), three arbitrary stimuli have been defined, with which it is possible to define all real colors. Through extensive color-matching experiments with human subjects having normal color vision, distributing coefficients have been measured for each visible wavelength (400 nm to 700 nm) giving the relative amount of stimulation of each receptor caused by light of that wavelength. These distribution coefficients $\bar{x}$, $\bar{y}$, $\bar{z}$, are shown below. Similarly, for any color the amount of stimulation of each receptor in the eye is defined by the set of *Tristimulus values* (X, Y, and Z) for that color.

The relationships between the distribution coefficient (see *accompanying figure*) and the tristimulus values are given in the equations

$$X = \int_0^\infty f_\lambda \bar{x}_\lambda P_\lambda d\lambda / Y',$$

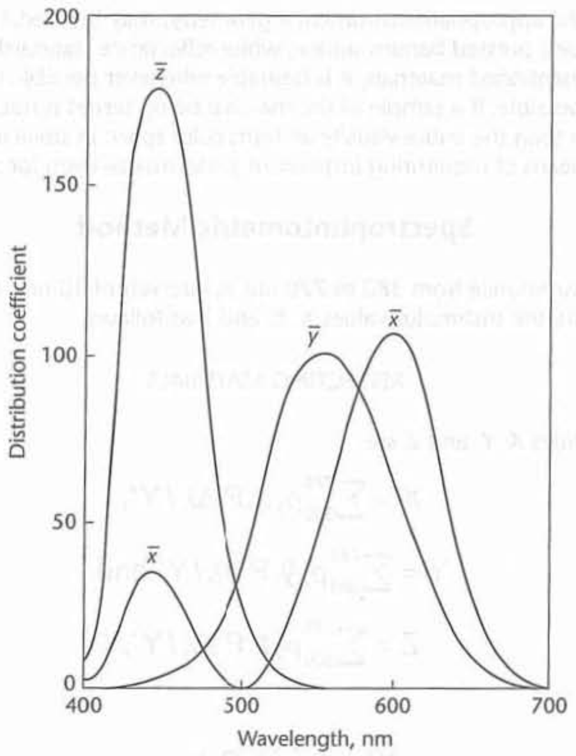
$$Y = \int_0^\infty f_\lambda \bar{y}_\lambda P_\lambda d\lambda / Y', \text{and}$$

$$Z = \int_0^\infty f_\lambda \bar{z}_\lambda P_\lambda d\lambda / Y',$$

in which

$$Y' = \int_0^\infty \bar{y}_\lambda P_\lambda d\lambda, P_\lambda$$

is the spectral power of the illuminant, and f_λ is either the spectral reflectance (ρ_λ) or spectral transmittance (τ_λ) of the material.



Distribution Coefficients from 400 to 700 nm

Once the tristimulus values of a color have been determined, they may be used to calculate the coordinates of the color in an idealized three-dimensional color space referred to as a *visually uniform color space.* Many sets of color equations have been developed in an attempt to define such a space. The equations given in this chapter represent a compromise between simplicity of calculation and conformance with ideality.

The coordinates of a color in a visually uniform color space may be used to calculate the deviation of a color from a chosen reference point. Where the instrumental method is used to determine the result of a test requiring color comparison of a test preparation with that of a standard or matching fluid, the parameter to be compared is the difference, in visually uniform color space, between the color of the blank and the color of the test specimen or standard.

PROCEDURE

The considerations discussed under *Ultraviolet-Visible Spectroscopy* ⟨857⟩ apply to instrumental color measurement as well. In the spectrophotometric method, reflectance or transmittance values are obtained at discrete wavelengths throughout the visible spectrum, a band width of 10 nm or less being used. These values are then used to calculate the tristimulus values through the use of weighting factors.[1] In the colorimetric method, the weighting is performed through the use of filters.

In the measurement of the spectral reflectance of opaque solids, the angle of viewing is separated from the angle of illumination in such a manner that only rays reflected diffusely from the test specimen enter the receptor. Specular reflection and stray light are excluded.

For the measurement of the spectral transmittance of clear liquids, the specimen is irradiated from within 5 degrees of the normal to its surface, and the transmitted energy measured is that confined within 5 degrees from the normal. The color of solutions changes with the thickness of the layer measured. Unless special considerations dictate otherwise, a layer 1 cm thick should be used.

The methods described here are not applicable to hazy liquids or translucent solids.

Calibration

For purposes of calibration, one of the following reference materials may be used, as required by instrument geometry. For transmittance measurements, purified water may be used as a white standard and assigned a transmittance of 1.000 at all wavelengths. Then the tristimulus values X, Y, and Z for CIE source C are 98.0, 100.0, and 118.1, respectively. For reflectance measurements, opaque porcelain plaques, whose calibration base is the perfect diffuse reflector and whose reflectance charac-

[1] Typical weighting factors are given by ASTM Z58.7.1-1951 as reported in the *Journal of the Optical Society of America*, Vol. 41, 1951, pages 431-439.

General Chapters

teristics have been determined for the appropriate instrumental geometry, may be used.[2] If the geometry of sample presentation precludes the use of such plaques, pressed barium sulfate, white reflectance standard grade, may be used.[3]

After calibration with the above-mentioned materials, it is desirable whenever possible to measure a reference material as close to the color of the sample as possible. If a sample of the material being tested is not suitable for use as a long-term standard, color chips are available[4] which span the entire visually uniform color space in small increments. The use of such a reference standard is encouraged as a means of monitoring instrument performance even for absolute color determinations.

Spectrophotometric Method

Determine the reflectance or transmittance from 380 to 770 nm at intervals of 10 nm. Express the result as a percentage, the maximum being 100.0. Calculate the tristimulus values X, Y, and Z as follows.

REFLECTING MATERIALS

For reflecting materials the quantities X, Y, and Z are

$$X = \sum_{380}^{770} \rho_\lambda \bar{x}_\lambda P_\lambda \Delta\lambda / Y',$$

$$Y = \sum_{380}^{770} \rho_\lambda \bar{y}_\lambda P_\lambda \Delta\lambda / Y', \text{and}$$

$$Z = \sum_{380}^{770} \rho_\lambda \bar{z}_\lambda P_\lambda \Delta\lambda / Y',$$

in which

$$Y' = \sum_{380}^{770} \bar{y}_\lambda P_\lambda \Delta\lambda \rho_\lambda$$

is the spectral reflectance of the material, $\bar{x}_\lambda P_\lambda$, $\bar{y}_\lambda P_\lambda$, and $\bar{z}_\lambda P_\lambda$ are known values associated with each Standard Source,[1,2] and $\Delta\lambda$ is expressed in nm.

TRANSMITTING MATERIALS

For transmitting materials, the quantities X, Y, and Z are calculated as above, τ_λ (spectral transmittance) being substituted for ρ_λ.

Colorimetric Method

Operate a suitable colorimeter[5] to obtain values equivalent to the tristimulus values, X, Y, and Z. The accuracy with which the results obtained from the filter colorimeter match the tristimulus values may be indicated by determining the tristimulus values of plaques of strongly saturated colors and comparing these values with those computed from spectral measurements on a spectrophotometer.

INTERPRETATION

Color Coordinates

The Color Coordinates, L*, a*, and b* are defined by

$$L^* = 116(Y/Y_0)^{1/3} - 16,$$

$$a^* = 500[(X/X_0)^{1/3} - (Y/Y_0)^{1/3}], \text{ and}$$

$$b^* = 200[(Y/Y_0)^{1/3} - (Z/Z_0)^{1/3}]$$

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[2] Suitable items are available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

[3] Suitable material is available from Eastman Kodak Company, Rochester, NY 14650, as "White Reflectance Standard."

[4] Centroid Color Charts may be obtained from suppliers of instruments for measurement of color.

[5] A suitable tristimulus colorimeter is available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

in which X_0, Y_0, and Z_0 are the tristimulus values of the nominally white or colorless standard, and $Y/Y_0 > 0.01$. Usually they are equal to the tristimulus values of the standard illuminant, with Y_0 set equal to 100.0. In this case $X_0 = 98.0$ and $Z_0 = 118.1$.

Color Difference

The total Color Difference ΔE^* is

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

in which ΔL^*, Δa^*, and Δb^* are the differences in color coordinates of the specimens being compared.

Instrumental variables can influence results. Although reliable comparisons can be made between similar colors measured concomitantly, results obtained on different instruments or under different operating conditions should be compared with caution. If it is necessary to compare data obtained from different instruments or taken at different times, etc., it is very helpful to have concomitant data obtained on a standard reference material such as color chips for opaque materials. Comparison of the readings on the reference material helps to identify variations caused by instrument performance.

⟨1063⟩ SHEAR CELL METHODOLOGY FOR POWDER FLOW TESTING

1. INTRODUCTION
   1.1 Scope
2. THEORY AND PRINCIPLES
3. DESCRIPTION OF SHEAR CELL COMPONENTS AND DESIGNS
4. SHEAR CELL MEASUREMENTS
   4.1 Sample Preparation
   4.2 Instrument Preparation
5. SELECTION OF TEST CONDITIONS
6. TEST PROCEDURE
7. DATA ANALYSIS AND CALCULATIONS
8. REFERENCE MATERIALS AND REPRODUCIBILITY

1. INTRODUCTION

A large number of pharmaceutical processes involve powder transfer and feeding, and the ability of powders to flow in a controlled manner during these operations is critical to product quality. For example, drug product attributes such as weight and content uniformity depend on powder flow characteristics. Shear cells are among the most important methods for measuring powder flow properties, and the data from shear cell testing can be used to predict a wide variety of powder flow behaviors during pharmaceutical manufacturing. Shear cells have many advantages over simpler methods of measuring powder flow (see *Powder Flow* ⟨1174⟩), but their operation is more complex and the procedures for their use must be carefully controlled to produce accurate and reproducible data. This chapter describes best practices for obtaining reliable and accurate powder flow data using a shear cell.

1.1 Scope

This chapter focuses on the three most popular shear cell types used for measuring powder flow properties:
1. Translational (Jenike type)
2. Annular (Schulze type)
3. Rotational (Peschl type)

These three shear cell types are categorized as direct shear tests in which a region of the powder is sheared under a series of controlled normal stresses. From these data, a wide variety of parameters can be obtained, including the yield locus representing the shear-stress to normal-stress relationship at incipient flow, the angle of internal friction, the unconfined yield strength, powder cohesion, and a variety of related parameters such as the flow function. In addition, these three shear cells can be set up with wall coupons (see *Appendix*) to measure the powder wall friction. When the shear cell data are combined, they can be used for bin and hopper evaluation and design. Other testing approaches, such as triaxial testers and indirect or hybrid testers (e.g., the Johanson indicizer), are outside the scope of this chapter.

2. THEORY AND PRINCIPLES

The flow behavior of a powder is fundamentally different from the flow of a fluid. First, powder flow properties and shear behaviors are strongly dependent on the consolidation stresses applied to the powder and are minimally dependent on the strain or flow rate (under the assumption of quasi-static conditions such as flow in a bin). Fluid flow, in contrast, is strongly dependent on the strain rate (where viscosity describes the relationship between shear stresses and strain rates) and is minimally dependent on absolute pressure. Second, when shear stresses are applied to powders, they may not immediately fail (i.e., they can avoid flow under a sustained shear stress), whereas Newtonian and viscoelastic fluids do not behave this way and always flow under an applied shear stress. Thus, powders have the potential for arching and rat-holing, depending on the flow pattern (see *Appendix*).

However, powders can sustain a shear stress without flowing only up to a certain point. The yield locus for a given powder is a function of many variables, including its composition, particle size and shape, moisture content, temperature, time stored at rest, and the state of consolidation. Once a powder is subjected to stresses (whether by gravity or some mechanical means) that reach or exceed the yield locus, the powder flows. Hence, determining the yield locus for a given powder under conditions representative of its manufacturing process is an essential step in evaluating the flow behaviors for that process. In some circumstances, this may involve testing under controlled environmental conditions, as well as holding the powder under load for an extended period before shearing (a "time test").

Because powder properties are highly dependent upon the degree of consolidation, the preparation of a uniform powder bed (consistent bulk density throughout the powder bed) is the first critical step of shear cell testing. The next stage of testing is the application of a normal stress (σ) and shear stress (τ) to the powder bed to achieve steady-state shear, resulting in a known state of consolidation. The shear stress then is removed, and a reduced normal stress is applied. A shear stress then is applied and is progressively increased until the powder bed yields and begins to flow. This procedure is repeated at several different normal stress conditions to create a "yield locus" plot. To complete a full flow function analysis, the operator must determine several yield loci, which requires that the unconfined yield strength be determined under several different levels of consolidation.

Although this chapter focuses on the powder (particle–particle) properties, the wall friction (particle–wall) properties and bulk density are also important. Such properties are used for bin design and also are essential when one compares different wall materials (e.g., different grades and finishes of stainless steel, or the effect of plastic coatings on powder flow behaviors). The most fundamental property of a wall material in this regard is Φ', the angle of friction between the bulk powder and the wall material, or, correspondingly, the coefficient of wall friction (μ_w):

$$\mu_w = \tan(\Phi') = \tau_w / \sigma_w$$

τ_w = wall shear stress
σ_w = wall normal stress

Note that Φ', and hence μ_w, often are a function of the applied normal stress (σ_w).

3. DESCRIPTION OF SHEAR CELL COMPONENTS AND DESIGNS

Figures 1, *2*, and *3* provide schematics of the different types of shear cells considered in this general chapter. All have the same general operating principle of being able to measure the force required to shear a powder bed to which a normal load has been applied. The applied load or measured force can be expressed as a stress by dividing by the cross-sectional area of the shear plane under consideration.

The translational shear cell (*Figure 1*) has a fixed base with a movable ring above it, and both hold the bulk powder. A cover that fits within the ring is used to contain the powder and to provide a uniform application of the normal load (N). The ring and cover are pushed as a unit while a load cell records the shear forces (F) that are generated. The shear plane forms between the powder that is contained in the base and the powder contained in the ring.

General Chapters

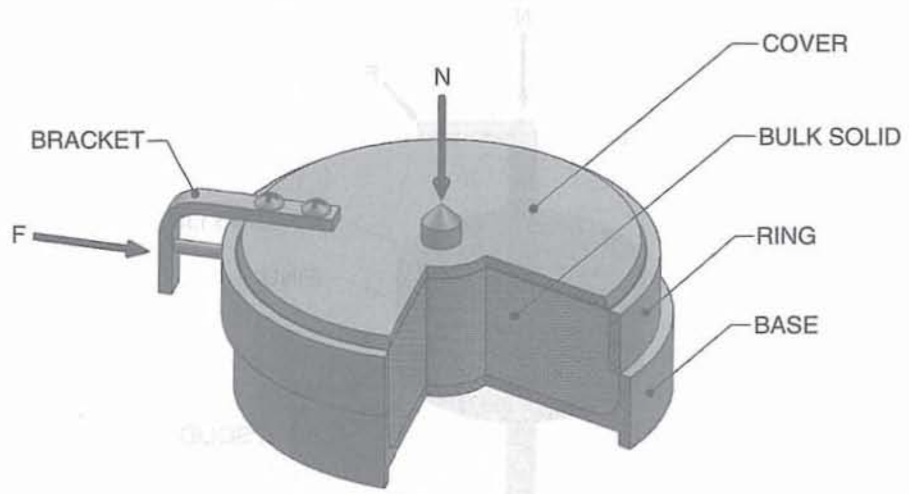

Figure 1. Descriptive schematic of the translational shear cell.

The annular shear cell (*Figure 2*) consists of a shear cell or base that holds the powder. A cover that fits within the cell is used to contain the powder and to provide a uniform application of the normal load (*N*). The cover is free to move up and down, but otherwise remains fixed in place by a load cell that measures the shear forces (*F*) that are generated. The shear cell is rotated at a constant angular velocity (ω) to create a shear plane that forms in the powder bed somewhere between the cell bottom and the cover. The cover and shear cell usually have baffles or other surface features that prevent the powder from sliding or shearing at the interface between the powder and the cover or shear cell base.

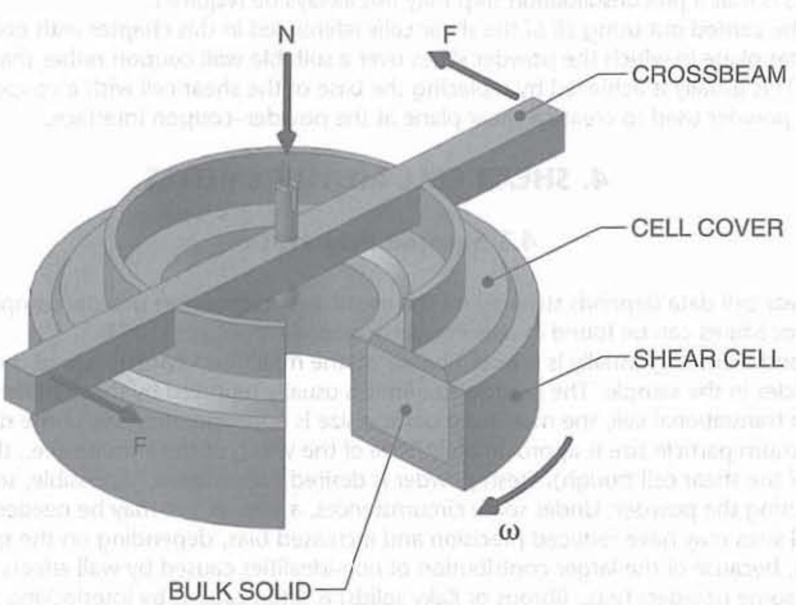

Figure 2. Descriptive schematic of the annular shear cell.

The rotational shear cell (*Figure 3*) has a base and ring that hold the powder. A loading lid that fits within the ring is used to contain the material and to provide a uniform application of the normal load (*N*). The loading lid is free to move up and down, but otherwise remains fixed in place, connected to a load cell that measures the shear forces (*F*) that are generated. The shear cell base then is rotated at a constant angular velocity (ω) to create a shear plane that forms in the powder bed somewhere between the ring and the base. Alternatively, the base can be fixed, and the lid can be rotated to create the shear plane in the powder specimen. The ring and base usually have surface features that prevent the powder from sliding at the powder-surface interface.

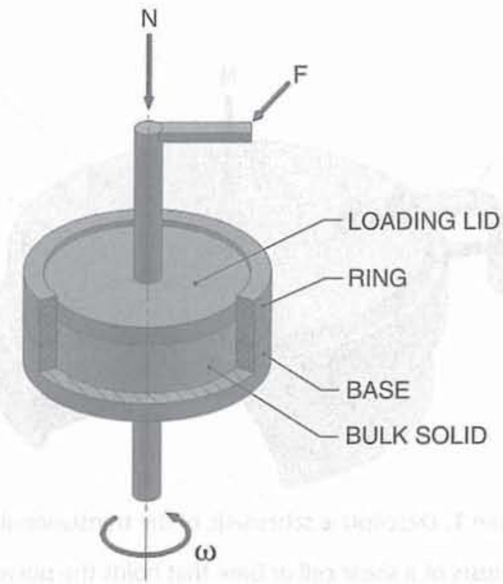

Figure 3. Descriptive schematic of the rotational shear cell.

The choice of one shear cell type over another depends on factors such as the availability of equipment and ease of use for a particular technician. Because of the infinite travel permitted with the annular and rotational shear cell geometry, these types of cells are more suited for powders for which reaching steady state requires large strains. Another advantage of the infinite travel of rotational testers is that a preconsolidation step may not always be required.

Wall friction tests can be carried out using all of the shear cells referenced in this chapter with only minor modifications, namely by creating a shear plane in which the powder slides over a suitable wall coupon rather than having the shear plane within the powder bed. This usually is achieved by replacing the base of the shear cell with a coupon of the wall material and adjusting the amount of powder used to create a shear plane at the powder–coupon interface.

4. SHEAR CELL MEASUREMENTS

4.1 Sample Preparation

The applicability of shear cell data depends strongly on the use of a representative powder sample. Detailed discussion of appropriate sampling procedures can be found in *Bulk Powder Sampling Procedures* ⟨1097⟩.

The cell size (volume and diameter) usually is selected based on the maximum particle size of the powder sample, and the proportion of large particles in the sample. The particle size limit is usually reported by the supplier of commercially available testing equipment. For a translational cell, the maximum particle size is approximately 5% of the diameter of the cell, and for an annular cell the maximum particle size is approximately 10% of the width of the annulus (i.e., the difference between the outer and inner radius of the shear cell trough). Fresh powder is desired for each test, if possible, so the use of a smaller cell may be preferable to reusing the powder. Under some circumstances, a smaller cell may be needed to reach higher consolidation stresses. Smaller cell sizes may have reduced precision and increased bias, depending on the specific tester and the cell size under consideration, because of the larger contribution of non-idealities caused by wall effects.

The shear strength of some powders (e.g., fibrous or flaky solids) is often caused by interlocking of particles; therefore, such materials may prove to be unsuitable for testing with a conventional shear cell. Likewise, powders with high springback (rubber-like or highly elastic properties) sometimes can fall outside of the practical limits of a shear tester. Poor reproducibility and unexpected results may indicate a problem with testing these types of powders.

Powder samples must be handled and tested under conditions that are relevant from a practical standpoint. For example, many pharmaceutical powders will sorb/desorb moisture from their surroundings according to the ambient relative humidity and temperature. This sorbed moisture can significantly affect the measured powder flow properties. Controlling the environmental conditions during sample handling and testing so that sample behavior is representative of the processing conditions of interest is essential for most powders.

Some pharmaceutical powders "age" quickly after they are produced, and such powders may need to be tested immediately after they are manufactured. The testing of some powders may result in caking or particle attrition that can render a powder unsuitable for retesting. In these circumstances, fresh powder samples should be used for each test.

4.2 Instrument Preparation

The shear cell should be situated in an area that is free of vibrations. Vibrations can affect the instrument readings and also can densify or dilate the powder while it is being tested.

Because shear testers measure forces, their load cells must be calibrated for the forces being measured. Some testers also measure the displacement (linear or angular) of the cell (indicating travel) and/or the vertical displacement of the cover or lid (used to calculate the volume of the cell and hence an average bulk density). In these cases, the displacement transducers must also be calibrated. The rate of any changes in force and displacement will need to be confirmed as part of this calibration. In addition, the shear cell is operated under the assumption that all of the parts are precisely aligned and there is a minimal degree of wobble when the cell rotates. For example, if the cover and shear cell trough are not parallel during testing, it will be difficult to get reproducible results. Thus, the alignment and mechanical operation of the shear cell must be evaluated at regular intervals. No other adjustments with respect to calibration are performed, although the correct performance of the apparatus may be confirmed by the measurement of the yield locus of a reference powder under standardized test conditions.

Wall coupons used for testing must be representative of the surfaces upon which the powder will slide. Directional surfaces can be oriented in the direction of flow in the application (e.g., grain oriented down the length of a hopper), oriented in the worst-case condition (often, grain oriented perpendicular to the direction of flow in the hopper), or evaluated in both directions.

All shear cell components (including wall coupons) should be carefully cleaned before use with a method that does not abrade the surface or leave any chemical residue behind. In addition, the cleaning method should be capable of removing all of the components of the test sample, including lubricants and other additives.

5. SELECTION OF TEST CONDITIONS

Preshear normal stresses usually are selected based on the powder's density values. Standards, such as ASTM Standard 6128, provide tables of specific initial preshear normal stresses as a function of the sample bulk density.[1] Subsequent preshear normal stresses are given in multiples of the initial preshear value (e.g., 2, 4, and 8 times the initial level). The number of preshear normal stress levels should be at least 4. It is often valuable to match preshear stresses in the test to the stresses expected in the processing situation of interest. Hold times for time tests are selected based on matching the hold time in the practical application. If long hold times are unrealistic, operators may consider several intermediate time points and extrapolation of the data.

Normal stress levels for shear are selected to provide a range of data points on the yield locus. Typically, a range between 25% and 80% of the preshear normal stress is valid, although powders with high unconfined yield strength or internal friction may require a narrower range. The range of normal shear stress levels should be sufficient to allow meaningful fitting and extrapolation of the data to determine the unconfined yield stress and other related parameters.

6. TEST PROCEDURE

Shear cell testing involves a sequence of steps that consolidates the powder to a known extent and then shears it under carefully controlled conditions while recording the applied normal stresses and measuring the shear stresses. In most cases, the essential steps are as follows:

1. Fill the test cell with an appropriate and representative sample of the powder in a manner that provides a uniform bulk density and composition. This sample in the test cell is referred to as the "specimen". Detailed procedures vary according to the type of tester being used, but in all cases it is essential to evenly distribute the powder and avoid pockets of air that can be difficult to remove later.
2. For translational shear cells, perform a preconsolidation step to create the desired density in the cell. This is accomplished by twisting the cover while under a compressive normal load. This step reduces the travel needed to achieve a steady-state preshear value and can limit the total vertical displacement of the cover during the test. A preconsolidation step may be conducted in other cell types, but this is often not conducted, because the unlimited rotation of the annular and rotational cells can generally provide a sufficient state of consolidation. The appropriate level of preconsolidation is critical, and care must be taken not to over- or under-consolidate the specimen.
3. Consolidate the specimen by applying a known normal stress to the powder specimen via the cell cover/lid.
4. Preshear the specimen until a steady-state shear value is reached. Care should be taken to avoid over-consolidating the specimen. The applied shear stress is then reduced to zero.
5. An instantaneous shear test is run by shearing the specimen under a reduced normal stress (with respect to the applied preshear stress) until the shear stress goes through a maximum value and then begins to decrease.

Steps 1–5 are repeated at a series of different reduced normal stress conditions to create a complete set of data (yield locus) and then at a series of different preshear normal stress conditions to create a "flow function". The test cell is preferably emptied and refilled before generating each point on the yield locus. For annular or rotational shear cell types, it is common that

[1] ASTM. D6128-06. Standard test method for shear testing of bulk solids using the Jenike shear cell. West Conshohocken, PA: ASTM; 2006.

steps 3–5 are repeated a number of times for the same specimen without emptying and refilling the cell. If the same specimen is used for multiple test points, caution must be taken to ensure that the specimen has not changed from test to test.

A wall friction test is run in an analogous manner by sliding the specimen over a coupon of wall material and measuring the shear stress as a function of the applied normal stress. In a time test, a normal stress is applied to the specimen for a predetermined period of time before shearing. Both wall-friction and time-consolidation tests can be conducted with the three types of shear cells described in this chapter, as long as directionality (if any) of the wall surface, relative to the cell movement, is taken into account.

7. DATA ANALYSIS AND CALCULATIONS

Illustrative results from the shear cell test procedure are presented in *Figure 4*. For analysis, the applied preshear normal stress (σ_p) and all valid applied normal stress points (σ_{si}) for $i = 1, 2, 3,..., n$ and their corresponding measured shear stress points[2] ($\tau_p, \tau_{s1}, \tau_{s2},..., \tau_{sn}$) are plotted in ($\sigma$, τ)-coordinates. These data are then used to generate a smooth line through all the valid shear points to obtain the yield locus. Typically, the yield locus passes above or through the preshear point. If not, the test results should be analyzed in more detail. In some circumstances, the yield locus can be forced to pass through the preshear point and can be fitted to all yield points.

The "unconfined yield strength" of the powder is obtained by drawing a Mohr circle through the origin and tangential to the yield locus. The higher point of intersection of this Mohr circle and the σ-axis is the unconfined yield strength f_c. The "major consolidation stress", σ_1, is found by drawing a second Mohr circle through the preshear point and tangential to the yield locus. The higher point of intersection of this Mohr circle (consolidation locus) and the σ-axis is the major consolidation stress.

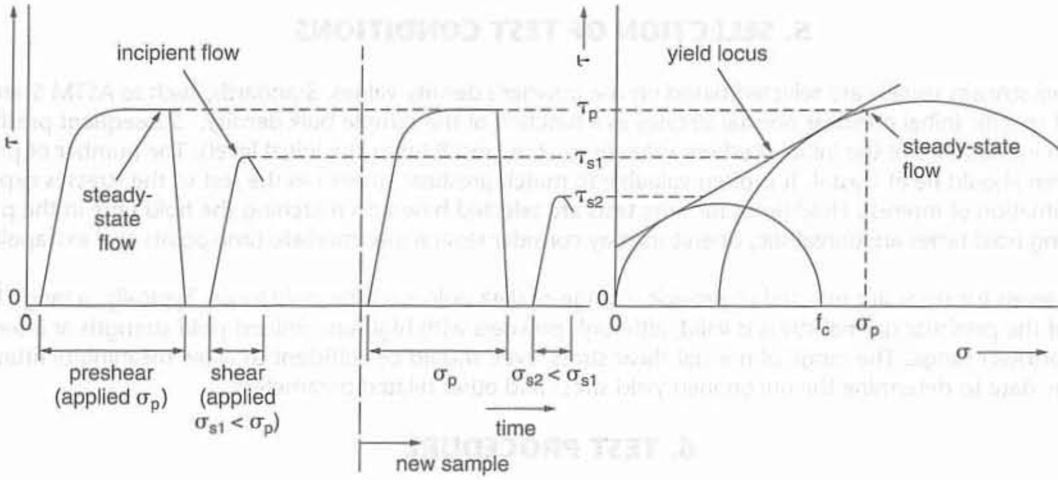

Figure 4. Shear cell testing and data analysis: shear cell raw data (*left*) and yield locus calculated from raw data (*right*).

The "angle of internal friction", Φ, is defined as the slope of the yield locus, as shown in *Figure 5*. A line drawn through the origin and tangential to the steady-state Mohr circle has an angle, δ, that is defined as the "effective angle of friction". The "cohesion," C, is the intersection of the yield locus and the τ-axis.

[2] For a given normal stress applied, the measured steady-state shear stress values should be consistent from test to test. To account for normal experimental variations, a given measured shear stress value may be prorated in an appropriate fashion, relative to the steady-state value associated with that point.

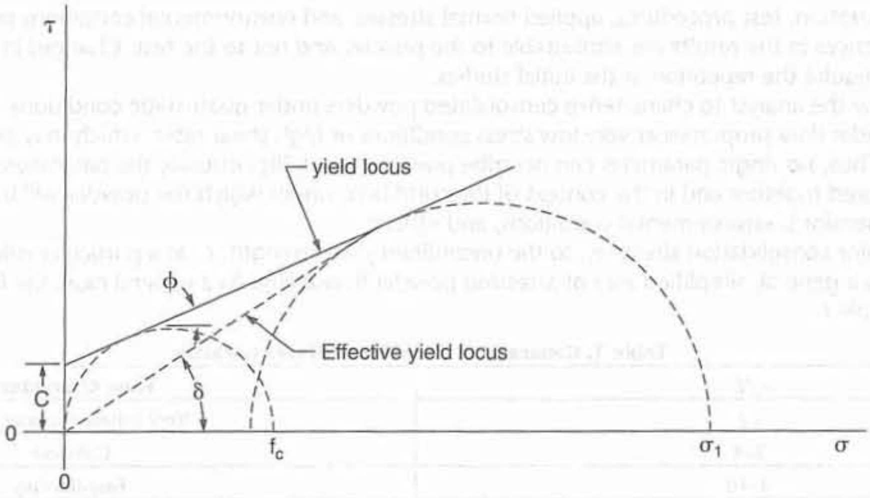

Figure 5. Graphical representation of the angle of internal friction (Φ), effective angle of friction (δ), and cohesion (C).

From a family of yield loci generated at different preshear normal stresses, it is possible to plot the unconfined yield strength, f_c, as a function of the major consolidation stress, σ_1, as shown in *Figure 6*. The best-fit curve to these points is called the "flow function" and can be used to calculate the potential for arching and rat-holing in a storage bin.

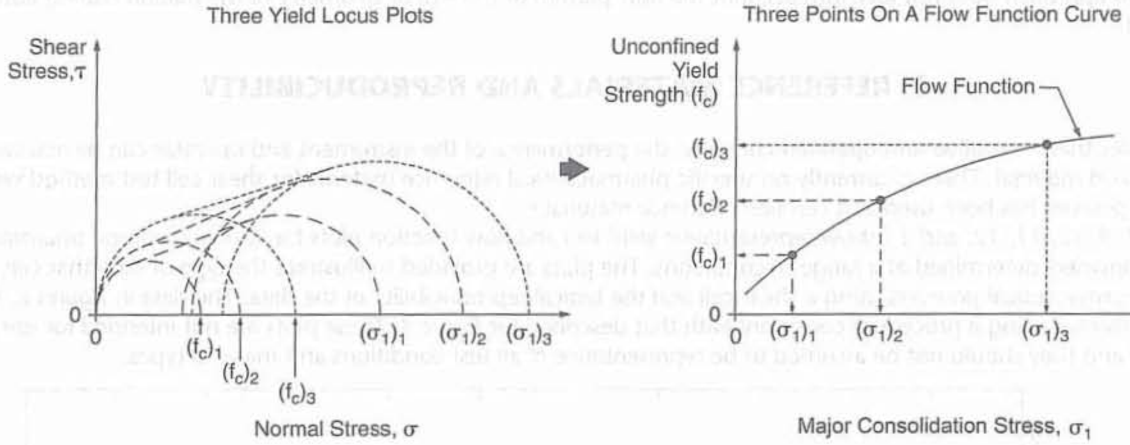

Figure 6. Calculation of the flow function from multiple yield locus plots.

The "kinematic wall yield locus" is developed in a manner similar to the calculation of the yield locus. This is depicted in *Figure 7*. The "wall friction angle", Φ', is defined as the inclination of a line from the origin to a point on the wall yield locus. In general, low wall friction angles are desirable.

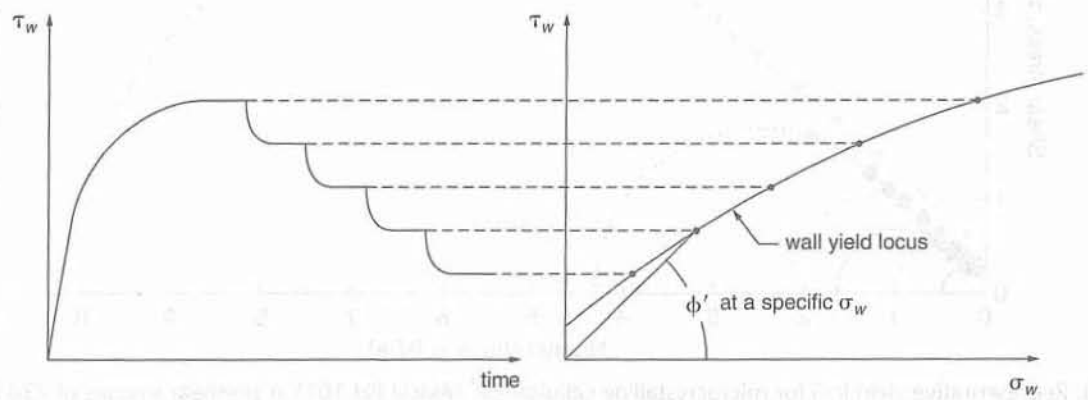

Figure 7. Calculation of the wall yield locus.

Test conditions should be carefully documented for every shear cell analysis to allow for accurate data interpretation and meaningful data comparisons. When analysts compare multiple powders, matching the tester type, specific tester model, test

General Chapters

cell size, sample preparation, test procedures, applied normal stresses, and environmental conditions provides increased confidence that any differences in the results are attributable to the powder and not to the test. Changes in material sourcing or manufacturing may require the repetition of the initial studies.

Shear cell tests allow the analyst to characterize consolidated powders under quasi-static conditions. The tests are unable to directly measure powder flow properties at very low stress conditions or high shear rates, which may occur in some powder-handling situations. Thus, no single parameter can describe powder flowability. Instead, the parameters described in this chapter should be interpreted together and in the context of the conditions under which the powder will be stored and handled (e.g., equipment dimensions, environmental conditions, and others).

The ratio of the major consolidation stress, σ_1, to the unconfined yield strength, f_c, at a particular value of the major consolidation stress provides a general, simplified way of assessing powder flowability. As a general case, the flow of powdered materials is classified in *Table 1*.

Table 1. General Classification of Flow Character[a]

σ_1/f_c	Flow Character
<2	Very cohesive, nonflowing
2–4	Cohesive
4–10	Easy-flowing
>10	Free-flowing

[a] Jenike A. Storage and flow of solids: bulletin no. 123 of the Utah Engineering Experiment Station. Salt Lake City, UT: University of Utah; 1964.

This approach can be useful for rank ordering the flow character of pharmaceutical powders as part of formulation and process development activities. However, it should be noted that this simplified view may lead to significant errors in interpretation because this approach does not take into account the flow pattern or the size or geometry of the manufacturing equipment being used.

8. REFERENCE MATERIALS AND REPRODUCIBILITY

For a tester that is installed and operated correctly, the performance of the instrument and operator can be assessed by testing a standard material. There is currently no specific pharmaceutical reference material for shear cell test method verification. Limestone powder has been used as a certified reference material.[3]

Figures 8, 9, 10, 11, 12, and *13* show representative yield loci and flow function plots for several common pharmaceutical excipient powders determined at a range of conditions. The plots are provided to illustrate the type of data that can be generated for pharmaceutical powders using a shear cell and the typical reproducibility of the data. The data in *Figures 8, 9, 10,* and *11* were collected using a procedure consistent with that described for *Figure 4*. These plots are not intended for use as reference data, and they should not be assumed to be representative of all test conditions and material types.

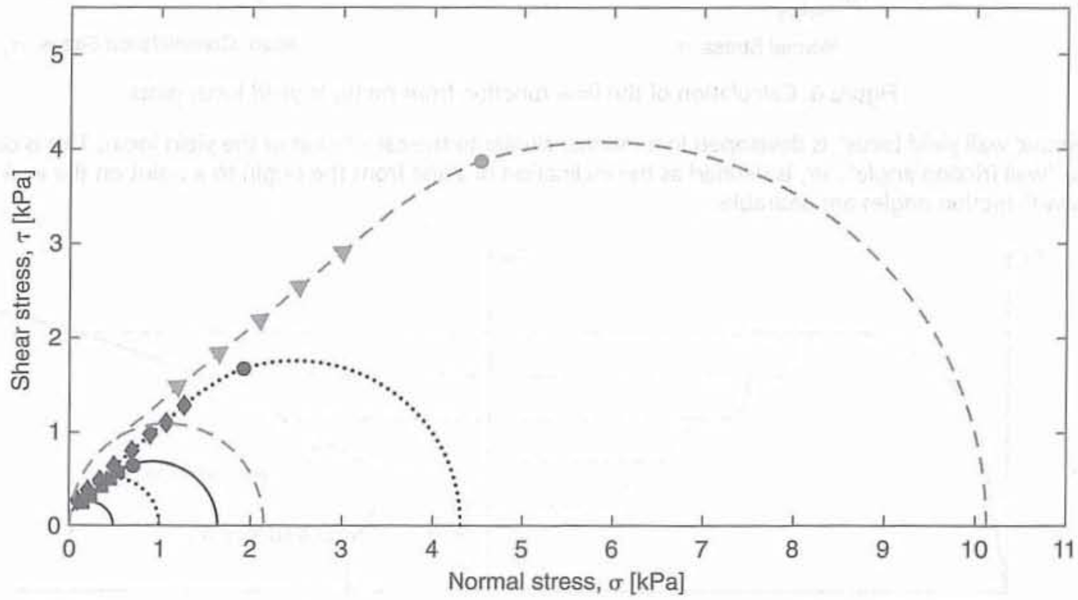

Figure 8. Representative yield loci for microcrystalline cellulose NF (Avicel PH-101) at preshear stresses of 730 Pa (*solid*), 1.9 kPa (*dot*), and 4.6 kPa (*dash*).

[3] Limestone powder certified reference material (BCR-116) is available from the Institute for Reference Materials and Measurements of the European Commission and instrument suppliers.

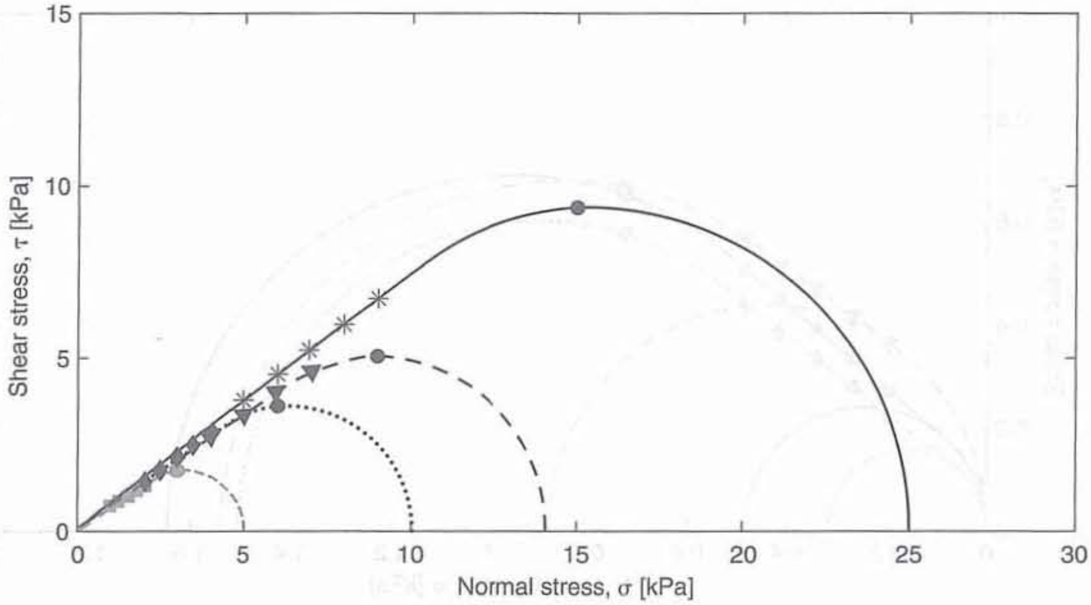

Figure 9. Representative yield loci for sorbitol at preshear stresses of 3 kPa (*gray, dash*), 6 kPa (*dot*), 9 kPa (*black, dash*), and 15 kPa (*solid*).

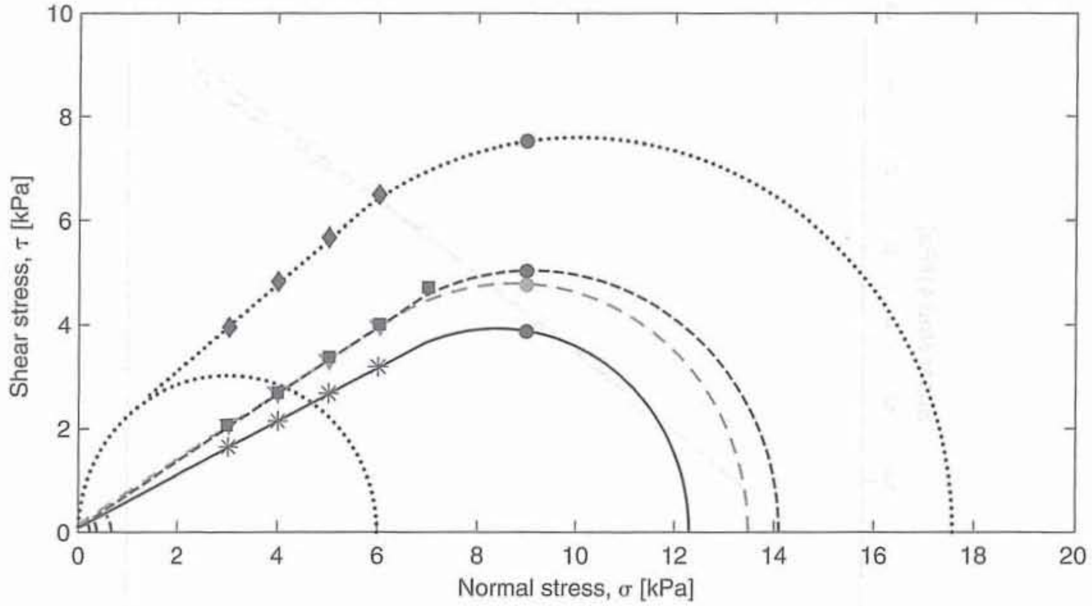

Figure 10. Representative yield loci for mannitol (*dot*), sorbitol (*black, dash*), sieved lactose (*gray, dash*), and spray-dried lactose (*solid*) at a preshear stress of 9 kPa.

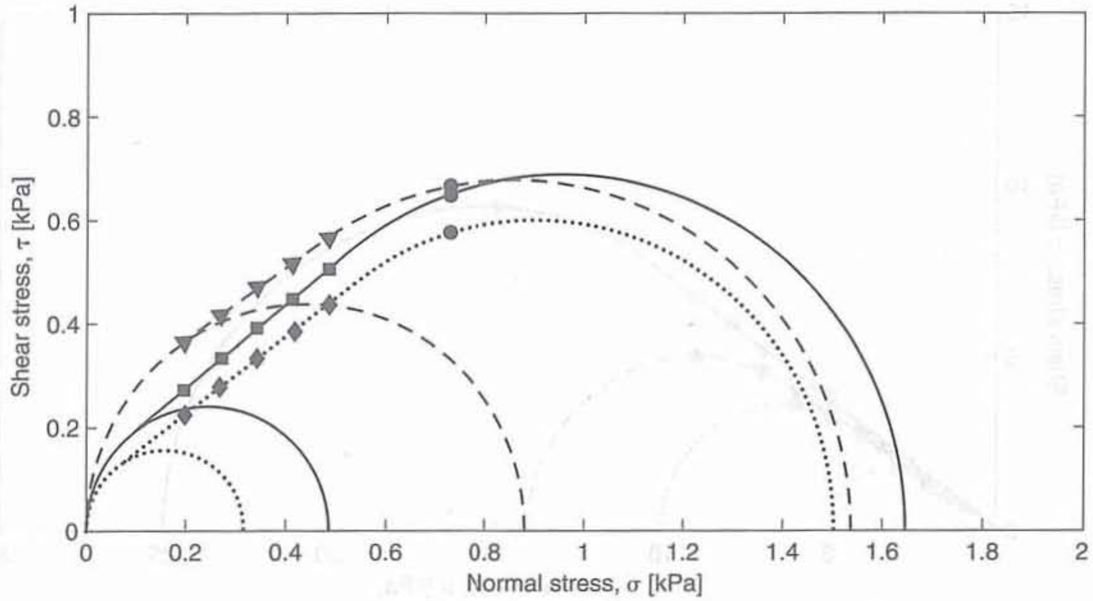

Figure 11. Representative yield loci for several different grades of microcrystalline cellulose NF. Avicel PH-101 (*solid*), Avicel PH-102 (*dot*), and Avicel PH-105 (*dash*) at a preshear stress of 730 Pa.

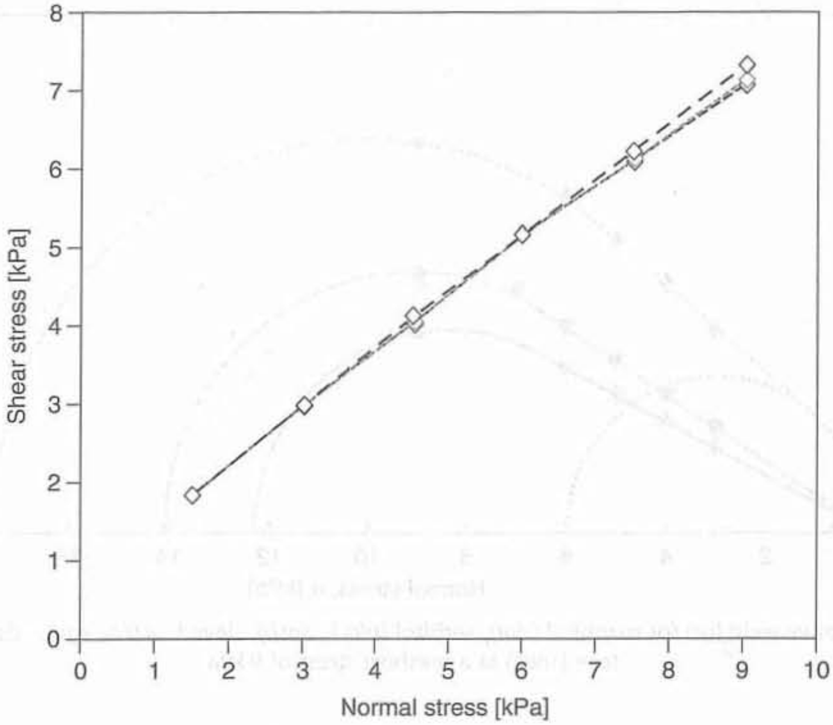

Figure 12. Representative yield loci for microcrystalline cellulose (Avicel PH102) from three independent determinations conducted at a preshear stress of 9 kPa. [Figure adapted from Sun CC. Setting the bar for powder flow properties in successful high speed tableting. *Powder Technol.* 2010;201(1):106–108.]

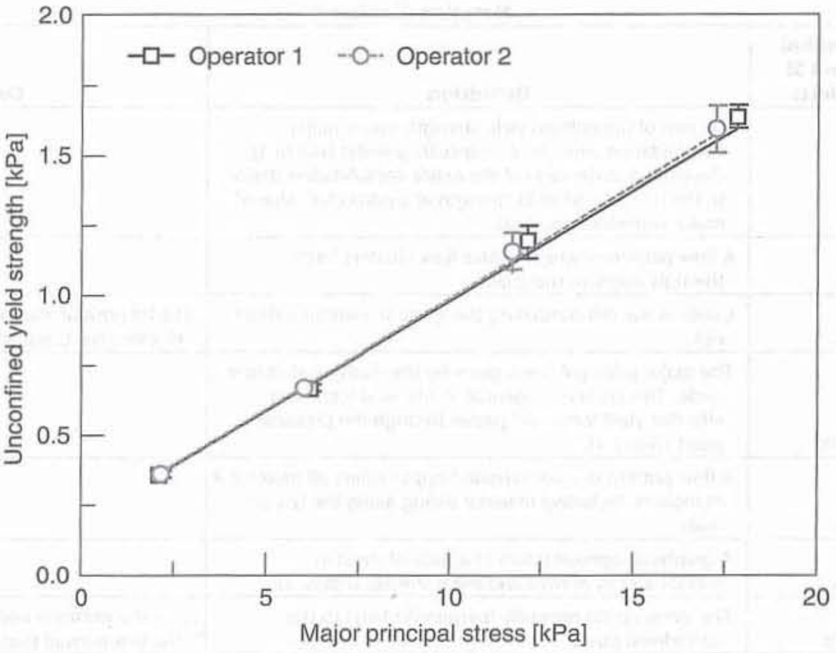

Figure 13. Representative flow function plots for microcrystalline cellulose (Avicel PH102) from two independent operators. [Figure adapted from Shi L, Chattoraj S, Sun CC. Reproducibility of flow properties of microcrystalline cellulose—Avicel PH102. *Powder Technol.* 2011;212(1):253–257.]

APPENDIX

Notation

Term	Symbol and SI Units	Definition	Comments
Angle of internal friction	Φ, degrees	The inclination of the yield locus (*Figure 5*).	
Angular velocity	ω, degrees per second	The angular velocity at which the shear cell base rotates.	Is usually held constant.
Annular shear cell	N/A	A shear cell based on a rotating annulus design (*Figure 2*).	The Schulze Ring Shear Tester is a common annular shear cell.
Arching	N/A	The formation of a bridge of powder across an opening caused by, for example, attractive interactions between particles.	Also known as "bridging".
Bulk density	ρ, kg/m^3	The mass of a quantity of a powder divided by its total volume.	Varies with the applied normal stress and history of a sample.
Cohesion	*C*, *Pa*	The failure shear stress at zero normal stress, normally obtained by extrapolation of the yield locus (*Figure 5*).	An indication of the intrinsic strength of an unconfined powder.
Consolidation	N/A	The process of increasing the density of a powder, which usually results in increasing its unconfined yield strength. Achieved in a shear cell by applying a preshear normal load and shear load to the specimen.	
Coupon	N/A	Flat surface that is in contact with powder specimen during wall friction testing.	The coupon surface finish must be representative of the wall surface of interest.
Effective angle of friction	δ, degrees	The inclination of the effective yield locus (*Figure 5*).	Sometimes used as a measure of relative flowability.
Effective yield locus	N/A	The straight line passing through the origin of the $\sigma-\tau$ plot and tangential to the steady-state Mohr's circle, corresponding to steady-state flow conditions of a powder of given bulk density (*Figure 5*).	
Flowability	N/A	A qualitative estimate of the relative flow properties of a powder.	Often based on measured values of the flow function or effective angle of friction.

Notation *(Continued)*

Term	Symbol and SI Units	Definition	Comments
Flow function	N/A	The plot of unconfined yield strength versus major consolidation stress for one specific powder (*Figure 6*). Quantified as the ratio of the major consolidation stress to the unconfined yield strength at a particular value of major consolidation stress.	
Funnel flow	N/A	A flow pattern where an active flow channel forms through stagnant material.	
Lid	N/A	Cover of the cell containing the specimen within a shear cell.	The lid position can be measured to determine displacement, bulk density, and powder dilation.
Major consolidation stress	σ_1, *Pa*	The major principal stress given by the steady-state Mohr circle. This circle is tangential to the yield locus and effective yield locus and passes through the preshear point (*Figure 4*).	
Mass flow	N/A	A flow pattern in a converging hopper where all material is in motion, including material sliding along the hopper walls.	
Mohr's circle	N/A	A graphical representation of a state of stress in coordinates of normal and shear stresses (*Figure 4*).	
Normal stress	σ, *Pa*	The stress acting normally (perpendicularly) to the considered plane.	σ_p is the preshear normal stress; σ_{si} for $i = 1, 2, 3,\ldots$ the test normal stresses.
Preconsolidation	N/A	Normal stress applied to consolidate a powder before testing in some shear cells (*Figure 4*).	
Preshear	N/A	Application of normal stress while shearing to attain steady-state shear conditions (*Figure 4*).	
Rat-holing	N/A	Tendency of powder to flow only in the region above an opening, thus forming a narrow rat hole in the powder.	Also known as "core flow".
Rotational shear cell	N/A	A shear cell operating under rotational shear conditions (i.e., rotational shearing perpendicular to the applied normal stress) (*Figure 3*).	The Peschl and Freeman shear cells are common rotational shear cells.
Shear stress	τ, *Pa*	The stress required to shear the powder in a direction perpendicular to the normal stress.	
Time consolidated strength	f_{ct}, *Pa*	The unconfined yield strength of a powder after being held at fixed consolidation conditions for a certain time.	
Time yield locus	N/A	The yield locus of a powder that has remained at rest under a given normal stress for a certain time.	
Translational shear cell	N/A	A shear cell operating under translational shear conditions (i.e., linear shearing perpendicular to the applied normal stress) (*Figure 1*).	The Jenike shear cell is a common translational shear cell.
Unconfined yield strength	f_c, *Pa*	The major principal stress of the Mohr stress circle that is tangential to the yield locus when the minor principal stress is zero (*Figure 4*).	
Wall friction angle	φ', degrees	The arctan of the wall friction coefficient (*Figure 7*).	
Wall friction coefficient	μ_w	The ratio of the wall shear stress to the wall normal stress at a given normal stress condition.	
Wall yield locus	N/A	A plot of the wall shear stress versus wall normal stress. Used to determine the wall friction angle for a powder-wall combination (*Figure 7*).	
Yield locus	N/A	A plot of shear stress versus normal stress at failure. The yield locus sometimes is called the instantaneous yield locus to differentiate it from the time yield locus (*Figure 4*).	A powder exhibits a series of yield loci for each different level of consolidation (density).

⟨1064⟩ IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY PROCEDURE

INTRODUCTION

Identification of botanical articles can be achieved by the application of multiple techniques, including macroscopic and microscopic descriptions, DNA analysis, and chemical means. *Identification of Articles of Botanical Origin* ⟨563⟩ provides a detailed discussion of these approaches. Chemical identification typically employs chromatographic or spectroscopic procedures to achieve the identification by fingerprint comparison against that of a Reference Standard, monograph description, or a reference chromatogram. Thin-layer chromatography (TLC) is one of the chromatographic techniques used in *USP* monographs for botanical articles in this way. High-performance thin-layer chromatography (HPTLC) is the most advanced version of TLC (see *Chromatography* ⟨621⟩), and a general analytical procedure for the application of HPTLC to the identification of botanicals is described in *High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin* ⟨203⟩. In HPTLC, the stationary phase consists of a uniform, typically 200-µm layer of porous (pore size 60 Å), irregular particles of silica gel with a size between 2 and 10 µm and an average particle size of 5 µm, plus a polymeric binder and a fluorescence indicator (F_{254}) coated onto a support, which is typically a glass plate or aluminum foil. Other stationary phases, such as chemically bonded phases (C8, C18, CN, NH2; DIOL) or microcrystalline cellulose, are also available with and without a fluorescence indicator. Because of the greater separation efficiency of the fine particles in HPTLC, the chromatographic system is miniaturized, using smaller developing chambers and shorter developing distances of 6–8 cm, in comparison to classical TLC, where 12–15 cm are required for best separation. As a consequence, less mobile phase and less time are required for chromatogram development. Another effect of miniaturization is the use of smaller sample volumes and the resulting possibility of analyzing more samples per plate than in classical TLC.

In addition, improved layer quality due to smaller particle size positively affects the signal-to-noise ratio, and therefore affects the detectability of separated sample components. A system suitability test is used to qualify the results. This feature is of great importance for the identification of botanical ingredients, which have an intrinsic natural variability in chemical composition. HPTLC features electronic images of chromatograms that allow convenient visual comparison of results obtained for multiple samples against images of chromatograms generated with reference materials. For purposes of identification, the complexity of botanical ingredients is best represented by fingerprints obtained in multiple detection modes from the same chromatogram, e.g., UV 254 nm and UV 366 nm light without derivatization, and white and UV 366 nm light after derivatization. Because compendial methods are used to determine compliance, variability in the results caused by the analytical method selected should be avoided. Because HPTLC can control the variables within narrow ranges using a rigorously standardized methodology and appropriate equipment, HPTLC is able to increase significantly the reproducibility of a chromatographic result from plate to plate compared to traditional TLC. Following here is a discussion of the variables to be controlled in order to achieve reproducible results in the identification of articles of botanical origin using HPTLC.

VARIABLES OF HPTLC

The plate is an open system affected by environmental factors, which must be controlled carefully. Unlike column chromatography, a closed system where samples are analyzed sequentially, in HPTLC multiple samples can be analyzed in parallel on the same plate. All steps of the planar chromatographic process are independent in time and location (an off-line process). For each of the steps—sample application, chromatogram development, visualization, detection, documentation, and evaluation—numerous parameters can be selected freely. This unique feature adds immense flexibility to the method and makes the planar chromatographic approach complementary to column-based techniques.

During method development, the many choices available for the various parameters can be overwhelming, and depending on the combination selected, there may be multiple results that all meet the respective analytical goal and yet are quite different. In this scenario, the labels "better" or "worse" are often assigned to the parameters according to personal preferences. In a cGMP (current Good Manufacturing Practices)-compliant environment, the reproducibility and integrity of results are of great importance. This is why the individual parameters of the HPTLC technique should be selected and combined thoughtfully in a standardized methodology so that the final result is optimized and can be compared to results obtained by different analysts. It may be important to avoid cumbersome and time-consuming steps, or to avoid the use of hazardous or toxic chemicals. Simplicity and clarity are desirable aspects of practical methods.

Handling of Plates

HPTLC plates are delicate and must be handled with care to avoid damaging the layer. When moving the plate, it should be touched only in the upper part above the region of chromatography. Each plate should be labeled with a soft pencil in the upper right (or left) corner. The expected developing distance can be marked on the right (or left) edge with a short pencil mark that goes down to the glass support. Marking the developing distance across the entire plate should be avoided because

it is difficult to judge when the rather-diffuse mobile phase front reaches that line. Aligning the front with a short mark on the edge is easier.

Plates should be stored in a place that is free of fumes and dust with the layer facing down to the stack. Shrink wrap of the package should not be in contact with the layer in order to avoid contamination with volatiles from the foil. Generally, HPTLC plates are ready for use without any pretreatment. Older or improperly stored plates may have accumulated impurities and therefore require precleaning. This is the case when after development, the solvent front (or additional secondary fronts) can be seen under UV 254 nm as an intense, broad dark band across the plate. Precleaning can be achieved by developing the plate in methanol to the upper edge. Subsequently, the plate is dried in a clean oven at 120° for 20 min. For cooling down to room temperature and for storage prior to further use, plates can be kept in an empty desiccator or wrapped in aluminum foil. Precleaning may slightly change the selectivity of a plate. Therefore, it is important to define in a method whether or not precleaned plates are to be used. Precleaning with the mobile phase is usually not a good option because it may be difficult to completely remove all components during the drying step.

Plate Layout and Sample Application

The standard format of the HPTLC plate is 20 × 10 cm (width × height). Other sizes (e.g., 10 × 10 cm) could be used as well, but one should keep in mind that for obtaining reproducible results, each size of plate requires a different chamber to maintain the same geometrical aspects as, for example, a twin-trough chamber would have for the standard plate (see *Chromatogram Development*). The optimum developing distance in HPTLC is 6 cm. Cutting plates to less than 10 cm in height provides no additional advantages.

Exact positioning is essential for proper identification of separated zones. For the application of samples with respect to plate layout (*Figure 1*), the following parameters must be considered.

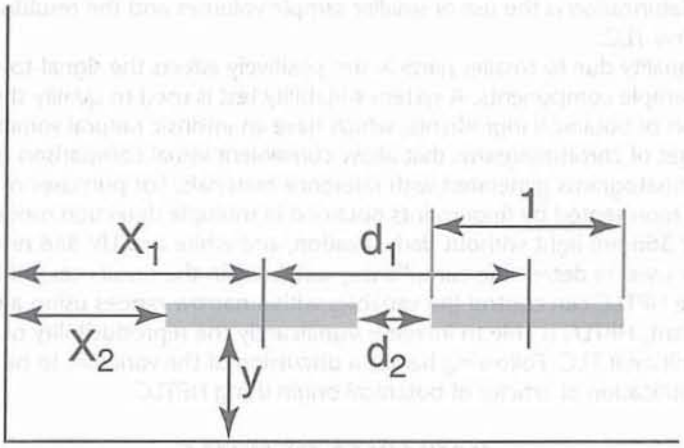

Figure 1. Plate layout.

For proper identification (migration distance/R_f values), all samples must be applied on a (virtual) line parallel to the lower edge of the plate. The distance (*y*) must be large enough to avoid the sample being immersed in the developing solvent. During development, the velocity of the mobile phase decreases with increasing developing distance. Consequently, the final result depends on the application position relative to the level of developing solvent (*Figure 2*).

General Chapters

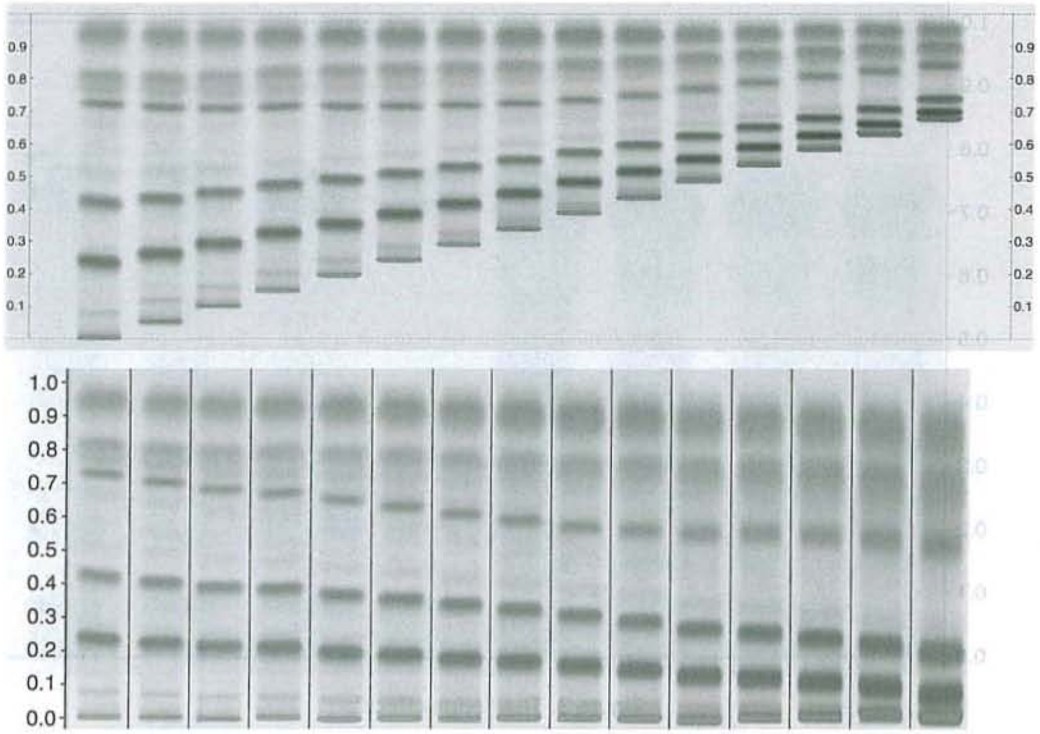

Figure 2. Upper part: Effect of the application position relative to the level of developing solvent (5 mm). Distance from lower edge ranges from 8–50 mm in increments of 3 mm. Lower part: R_F values calculated for the different effective developing distances. Sample is Houttuyniae herba; developing solvent is a combination of ethyl acetate, formic acid, and water (15:1:1, v/v/v); derivatization is Natural Products reagent (NP); detection is UV 366 nm.

For reproducible results, the distance from the lower edge (y) must be kept constant, and the amount of developing solvent placed into the chamber must also be fixed. To minimize solvent consumption, twin-trough chambers are used. The level of developing solvent in such chambers is typically 5 mm. A meniscus is formed on the plate at the immersion line. A sample applied at y = 8 mm will be well above this meniscus.

The distance of the application position from the left and right edges of the plate (x) must be sufficient to avoid the so-called "edge effect" (*Figure 3*). During production, most precoated plates develop a small rim on their edges where the layer is slightly thicker. This causes the mobile phase to advance faster, and samples that are applied directly onto or too close to the rim will migrate unevenly.

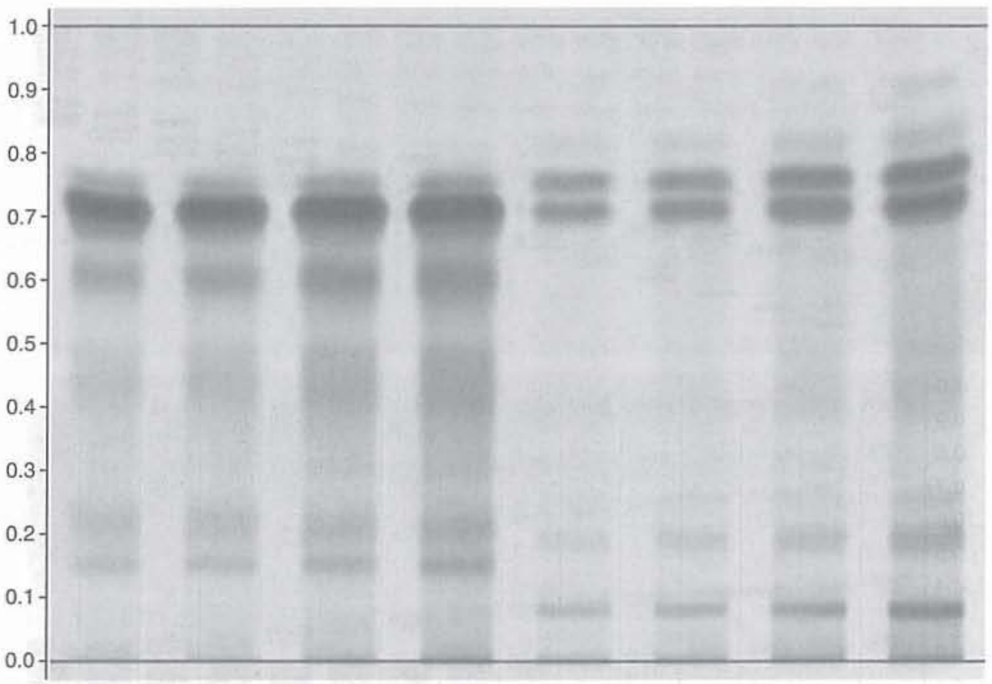

Figure 3. The "edge effect".

The position x_1 is typically called "application position". In order to avoid the edge effect, the length of the applied band (*l*) has to be taken into account when defining x_2 as the distance from the edge of the plate. A minimum of 15 mm is necessary for x_2. The distance between two samples (d_1) also considers the band length (*l*). For d_2, a minimum of 3 mm is recommended so that samples do not interfere with each other if larger volumes are applied. The band length (*l*) must be fixed for each method because it affects the concentration of samples per band if a defined volume of sample solution is applied. The applied quantity (across a defined band length) has an effect on the intensity of the separated zones and may affect whether or not a particular zone of a fingerprint is visible. An argument for selecting shorter bands would be that more samples can be applied on the plate. However, because chromatogram evaluation is primarily performed visually, it has to be noted that a band seems to look "sharper" (more like a band and less oblong) when it is >5 mm (*Figure 4*). A band length of 8 mm is a good compromise also from the perspective that ⟨621⟩ specifies bands of 5–10 mm for HPTLC plates.

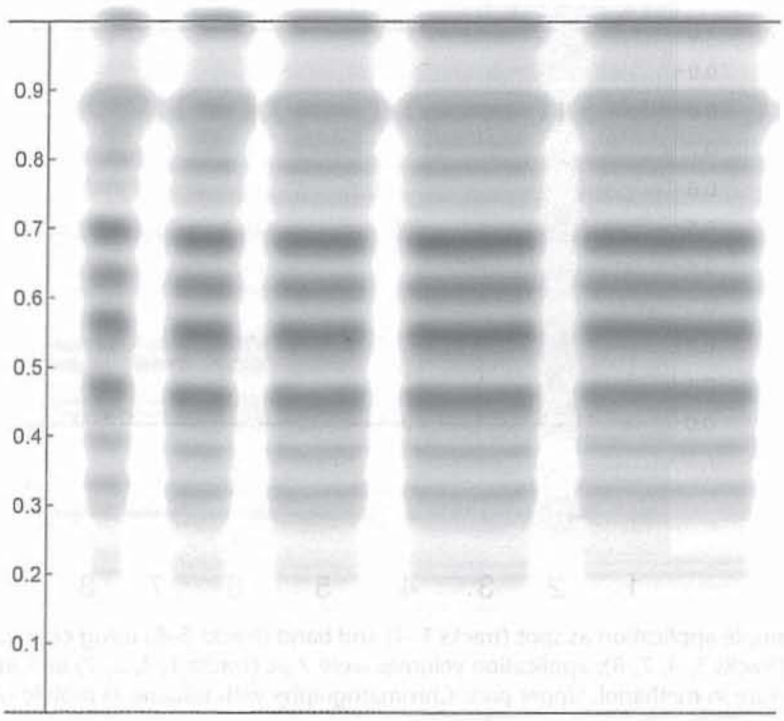

Figure 4. Effect of band length on the visual impression of separation. From left to right, 3 µL/3 mm, 5 µL/5 mm, 8 µL/8 mm, 11 µL/11 mm, and 15 µL/15 mm. Sample is Tiliae flos; developing solvent consists of ethyl acetate, formic acid, water, and methyl ethyl ketone (50:10:10:30, v/v/v/v); derivatization is NP/Polyethylene glycol reagent (PEG); detection is at UV 366 nm.

With the parameters just discussed, the standard plate layout will feature 15 bands of 8-mm length. If fewer samples are to be analyzed, it is recommended to either apply replicates or leave some tracks on the plate blank.

During application, samples are precisely deposited onto the HPTLC plate at defined positions and in defined quantities. Sample solutions can be applied by contact using a capillary or a syringe, or by spraying the sample solution without touching the plate (spray-on technique). During contact application, the sample's solvent can perform circular chromatography (*Figure 5*). Therefore, only small volumes should be applied in one stroke, and if possible, only nonpolar solvents should be used. During spray-on application, the sample's solvent is nebulized and evaporated, creating sharp zones regardless of the polarity of the solvent.

If samples are applied manually, a spotting guide can be used for proper positioning. Marking the application position with a pencil is not a good option, because the layer can be damaged easily.

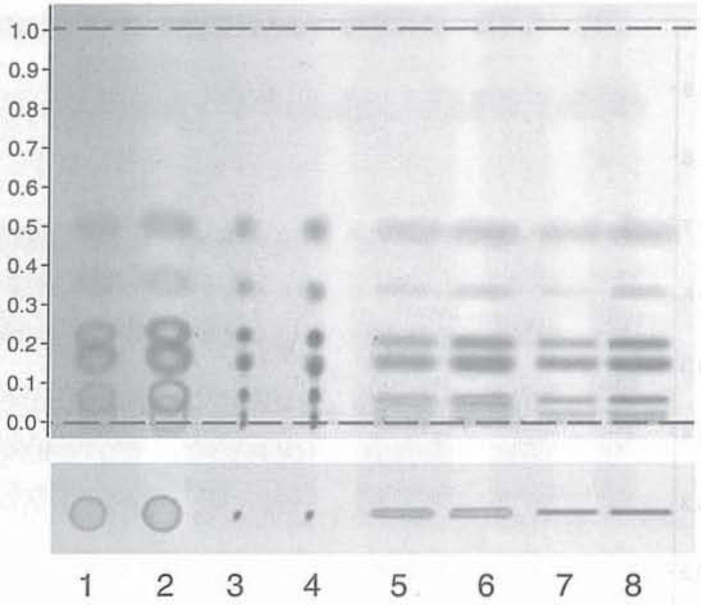

Figure 5. Lower part: Sample application as spot (tracks 1–4) and band (tracks 5–8) using contact application (tracks 1, 2, 5, 6) or spray-on technique (tracks 3, 4, 7, 8); application volumes were 2 µL (tracks 1, 3, 5, 7) or 5 µL (tracks 2, 4, 6, 8); test dye mixture in methanol. Upper part: Chromatography with toluene as mobile phase.

Chromatogram Development

The processes in a chromatographic chamber are highly complex and difficult to describe. Unlike column chromatography, which is assumed to take place in equilibrated chromatographic systems, planar chromatography always begins in a state of nonequilibrium and never reaches equilibrium. The sample has been applied onto the plate, which becomes the stationary phase only when it comes in contact with a liquid in the chromatographic chamber. The advancing mobile phase must be able to dissolve the sample for chromatography to begin. Driven by capillary action, the mobile phase velocity decreases with increasing migration distance because the resistance of the stationary phase against the flow is also increasing.

It can be shown experimentally that the optimum migration distance for mobile phases on HPTLC plates is about 6 cm. Depending on the viscosity of the mobile phase, development takes place between 10 and 20 min. To further move the solvent front just 10 mm will extend the developing time by 5–15 min, while moving an additional 20 mm will increase the developing time by 15–40 min. The contribution of that extra developing distance to improved separation is usually not justifiable, because the diffusion that occurs at decreased velocity of the mobile phase often offsets any gain in distance of separated zones.

Four distinct processes occur in a chromatographic chamber. These processes always take place simultaneously and affect each other (*Figure 6*).

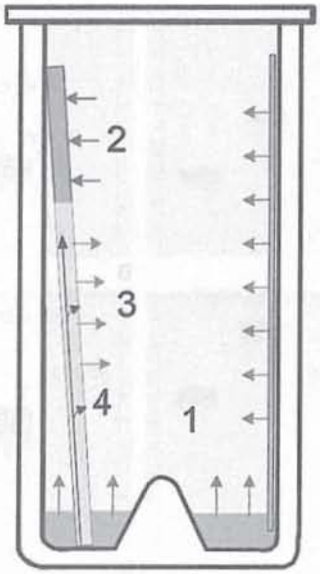

Figure 6. Processes occurring in the chromatographic chamber (twin-trough chamber): (1) chamber saturation; (2) plate preconditioning; (3) evaporation; (4) formation of secondary fronts.

Chamber saturation (*Figure 6*, process 1) is usually established before placement of the plate into the chamber. The developing solvent, which has a known composition, is placed into both troughs of the chamber, and the filter paper in the rear trough is wetted thoroughly. Chamber saturation occurs once the liquid in the tank is in equilibrium with its own vapor, and this is a function of time. After 20 min, the chamber will generally achieve a reproducible degree of saturation.

The plate should be placed into the chamber so that it rests in a vertical position against the front wall while the layer faces the rear wall. The dry portion of the layer absorbs solvent vapor from the gas phase (process 2) in order to achieve adsorptive saturation, which occurs when the surface is covered by a layer of solvent molecules. Generally, this process can lower R_f values and also affect the selectivity of the separation. In the lower portion of the plate, the liquid in the chamber forms the mobile phase. Depending on the degree of saturation, a part of the mobile phase may evaporate off the plate (process 3). Multicomponent mobile phases may be separated by the stationary phase because of different interactions of the constituents. This process is affected by the degree of preconditioning.

In an unsaturated chamber, the situation is quite different. The rear trough does not contain liquid or filter paper. The plate is introduced immediately after the solvent, which does not allow enough time to achieve gas–liquid saturation equilibrium. Preconditioning has little effect, while evaporation and possibly formation of secondary fronts (process 4) dominate the chromatographic process. Both of these processes typically cause alterations in the chromatograms, thereby affecting the separation.

In conclusion, each chamber gives a somewhat different result, depending on the chamber's geometry, the presence or absence of liquid and filter paper in one of the troughs, and the waiting time before introducing the plate. The chromatograms shown in *Figure 7* were obtained with an automatic developing chamber with humidity control. In this example, a saturated chamber (A) gives better separation than an unsaturated chamber (C). Chromatograms are reproducible if all conditions are the same (A) and (B). If the filter paper for saturation is not exchanged but is reused without drying, a completely different result is obtained (D).

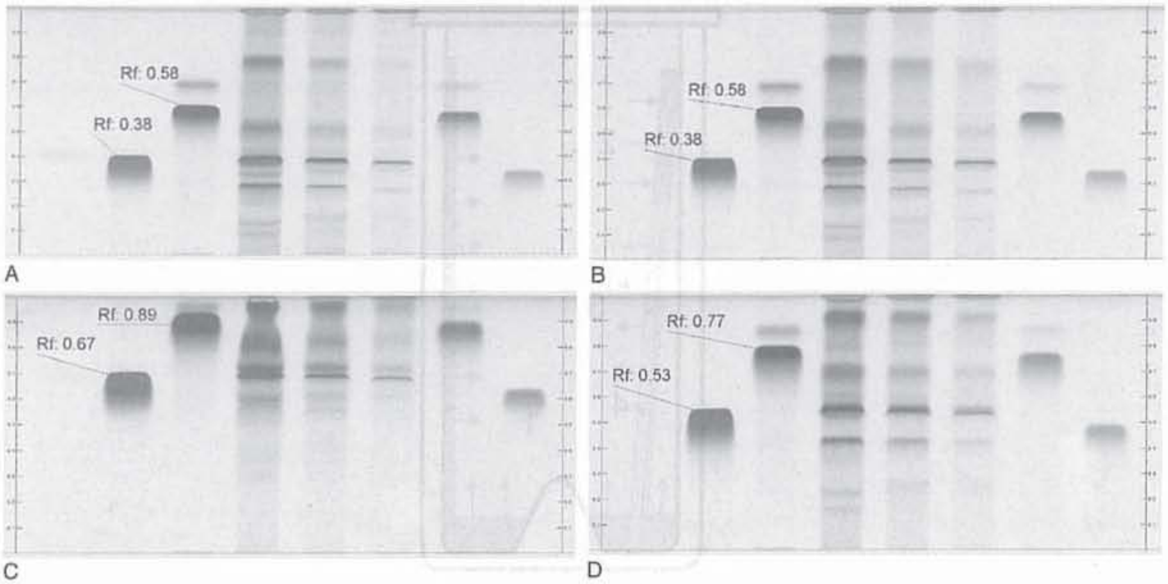

Figure 7. Effects of different chamber configurations at relative humidity 33%: (A) and (B) are saturated, (C) is unsaturated, and (D) is saturated with reused filter paper. Sample is *Lapsana communis*, references are chlorogenic acid (R_F 0.38, Lane 1, Panel A), quercitrine (R_F 0.58, Lane 2, Panel A), developing solvent: water, methanol, glacial acetic acid, and methylene chloride (2:3:8:15, v/v/v/v); derivatization NP/PEG; detection UV 366 nm.

For reproducible results, it is necessary to carefully standardize the development step. Chamber saturation is easy to achieve, as is proper timing. Unsaturated chambers are extremely sensitive to small changes and therefore, from a practical point of view, they should be avoided, even if they can give "better" separation. It is a good practice to keep developing distances constant (at optimum) for all methods, because other parameters, such as selectivity and condition of the gas phase, affect separation to a much greater degree.

Drying

After the mobile phase has advanced the desired distance, the plate must be removed from the chamber and reproducibly dried. This is best achieved by exposing the plate in the vertical position to a flow of cold air. Elevated temperature during drying could cause volatile sample components to diffuse or evaporate from the plate. During the evaporation of the mobile phase, sample components move from deeper areas of the layer to the surface. Uneven drying across the plate may result in different intensities for zones with equal amounts of substances.

Effects of Humidity

Silica gel has an extremely high affinity for water and is an effective drying agent. An HPTLC plate is always at equilibrium with the water vapor in the laboratory atmosphere (relative humidity, RH). Chromatographic results are affected by variation in the amount of water adsorbed onto the silica gel surface. The higher the RH, the more water is adsorbed. In a first approximation, this reduces the "activity" of the stationary phase and causes higher R_F values. Also, because the adsorbed water can become part of the stationary phase, the selectivity of the chromatographic system often changes in response to variation in RH.

Unfortunately, it is nearly impossible to predict what effect a change in RH may have for a given separation (*Figure 8*). Some separations are more affected than others, and sometimes only parts of the chromatogram will change. In addition, there is no ideal activity of the plate that can solve all separation problems, and it is difficult to use defined activity changes of the stationary phase to influence separation in a desired way. The best option is to keep the RH constant at a moderate level (e.g., 33%) and then, try to adjust the selectivity of the mobile phase. One practical exemption is effective "drying off" of the plate using a molecular sieve. Activating a plate by heat (120° for 20 min) is possible, because under these conditions water adsorbed onto the silica gel is effectively removed, but when the plate is cooled down and stored, or during sample application it re-equilibrates to ambient relative humidity.

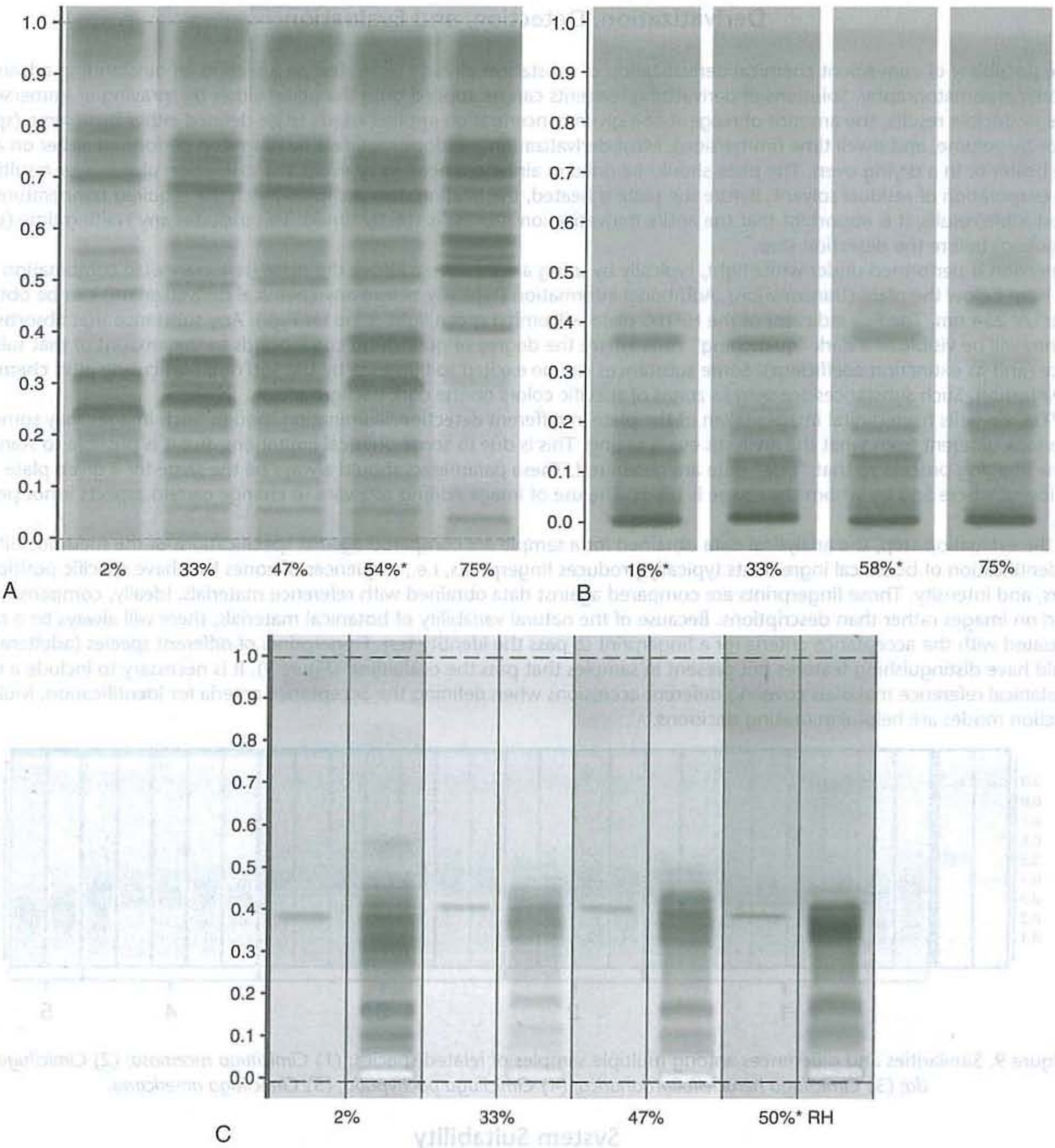

Figure 8. Effects of relative humidity on the separation of different samples. (A) *Hoodia gordonii,* developing solvent: chloroform, methanol, and water (70:30:3, v/v/v); derivatization: anisaldehyde reagent, detection white light. (B) Licorice root, developing solvent: ethyl acetate, formic acid, acetic acid, and water (15:1:1:2, v/v/v/v); derivatization: sulfuric acid reagent, detection white light. (C) Actein/Black cohosh rhizome, developing solvent: toluene, ethyl formate, and formic acid (5:3:2, v/v/v); derivatization: sulfuric acid reagent, detection UV 366 nm. Humidity: *ambient; 2%: molecular sieve; 33%: saturated solution of magnesium chloride ($MgCl_2$); 47%: saturated solution of potassium thiocyanate (KSCN); 75%: saturated solution of sodium chloride (NaCl).

To achieve reproducible results independent of seasonal or regional changes in the RH in various laboratories, it is important to expose the plate to a defined RH before development and with the samples already applied. This will keep the activity and the selectivity of the stationary phase constant. Defined RH can be created in closed containers such as a desiccator over saturated salt solutions. Between 20° and 25°, saturated solutions of magnesium chloride establish 33% RH; of potassium thiocyanate (KSCN), 47% RH; and of sodium chloride (NaCl), 75% RH.

Derivatization, Detection, and Evaluation

The possibility of convenient chemical derivatization of substances already separated on a plate is an outstanding advantage of planar chromatography. Solutions of derivatizing reagents can be applied onto the plate, either by spraying or immersion. For reproducible results, the amount of reagent of a given concentration applied needs to be defined either by volume (spraying) or by volume, and dwell time (immersion). Most derivatization reactions require a heating step performed either on a plate heater or in a drying oven. The plate should be dried in air before heating to avoid any disruption of the layer resulting from evaporation of residual solvent. Before the plate is heated, the heating device should reach the required temperature. For reproducible results, it is important that the entire derivatization process is strictly timed. This includes any waiting time (e.g., for cooling) before the detection step.

Detection is performed under white light, typically by using a light source above the plate (reflectance) in combination with light from below the plate (transmission). Additional information (typically before any chemical derivatization) can be obtained under UV 254 nm. The F_{254} indicator of the HPTLC plate will emit a green light (blue for F_{254s}). Any substance that absorbs UV 254 nm will be visible as a dark "quenching" zone where the degree of quenching corresponds to the amount of that substance (and its extinction coefficient). Some substances can be excited to fluoresce by UV 366 nm, particularly after chemical derivatization. Such substances are seen as zones of specific colors on the dark background.

HPTLC benefits from digital images taken of the plate in different detection/illumination modes. Such images may sometimes look different from what the analyst's eye is seeing. This is due to some physical limitations, but it is possible to standardize the imaging process so that "true" data are generated. These parameters should always be the same for a given plate regardless of where and by whom the image is taken. The use of image editing software to change certain aspects is not permissible.

In the evaluation step, the analytical data obtained for a sample are compared against specifications of the method. HPTLC for identification of botanical ingredients typically produces fingerprints, i.e., sequences of zones that have specific positions, colors, and intensity. Those fingerprints are compared against data obtained with reference materials. Ideally, comparison is based on images rather than descriptions. Because of the natural variability of botanical materials, there will always be a range associated with the acceptance criteria for a fingerprint to pass the identity test. Fingerprints of different species (adulterants) should have distinguishing features not present in samples that pass the evaluation (*Figure 9*). It is necessary to include a range of botanical reference materials covering different accessions when defining the acceptance criteria for identification. Multiple detection modes are helpful in making decisions.

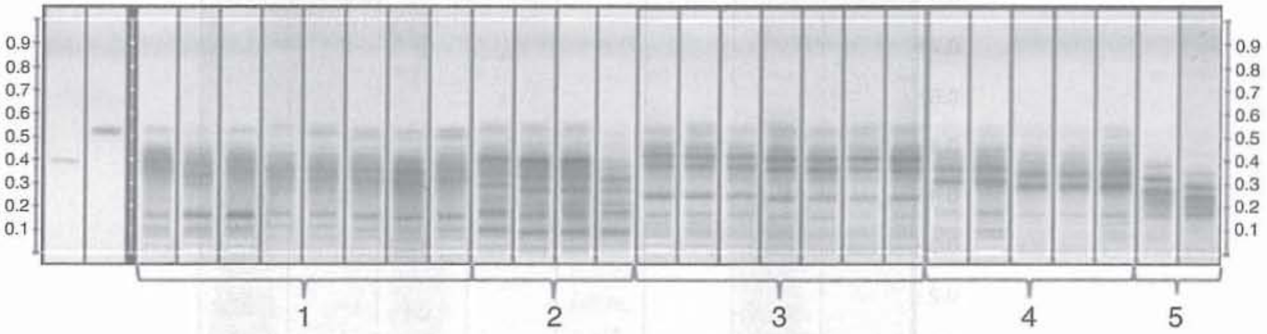

Figure 9. Similarities and differences among multiple samples of related species: (1) *Cimicifuga racemosa*; (2) *Cimicifuga foetida*; (3) *Cimicifuga heracleifolia/dahurica*; (4) *Cimicifuga pachypoda*; (5) *Cimicifuga americana*.

System Suitability

Before evaluating and/or comparing data that originate from different HPTLC plates, each developed plate must be qualified. This is possible with a system suitability test, which is part of the analytical method. On each plate, the analyst selects two or more reference substances that have similar but just separable R_F values under the chromatographic conditions to be used [e.g., chlorogenic acid (blue) and hyperoside (yellow-orange) in chromatographic systems used for flavonoids]. The substances designated to check the system suitability for resolution, position, and colors of the bands may be included in reference standard extracts or another matrix. Description of the resolution position and colors for the key bands of the reference material fingerprint should match the description in the reference within a specified tolerance range. Only when the system suitability requirements have been satisfied can the results obtained with the samples on the same plate be evaluated further to determine compliance. For reference to a standardized procedure for identification of articles of botanical origin controlling the variables described in this chapter, see ⟨203⟩.

CONCLUSIONS

As HPTLC is widely used for botanical identification to monitor the quality of articles of botanical origin on an increasingly globalized level, it seems important to thoroughly standardize the involved analytical procedures. All parameters discussed above must always be selected for each HPTLC analysis. Currently laboratories in different countries can make this selection widely within the framework of the *USP* general chapters; however, guidance about how to achieve specific HPTLC results in a reproducible manner is not available. A standardized procedure as proposed in ⟨203⟩ could fill this gap and serve as basis for harmonization of results obtained in different laboratories.

⟨1065⟩ ION CHROMATOGRAPHY

INTRODUCTION

Ion chromatography (IC) is a high-performance liquid chromatography (HPLC) instrumental technique used in USP test procedures such as identification tests, assays, and determination of impurities, including limit test and quantitative tests. IC is used to measure anions and cations derived from organic or inorganic molecules, from small molecules to larger biomolecules. This may include, but is not limited to, organic acids, carbohydrates, sugar alcohols, aminoglycosides, amino acids, amines, phosphonates, peptides, aminoglycosides, oligosaccharides, proteins, and glycoproteins.

IC has been applied to all aspects of the manufacturing and disposition of pharmaceutical products, including characterization of active ingredients, excipients, degradation products, impurities, and process streams. Raw materials, intermediates (including media and culture broths), bulk active ingredients, diluents, formulated products, production equipment cleaning solutions, process water, and waste streams may be analyzed using IC.

The majority of IC methods use either anion- or cation-exchange chromatography coupled with suppressed conductivity detection. IC is especially valuable for ionic or ionizable (in the mobile phase) analytes that have little or no native UV absorbance. In addition to suppressed conductivity detection, the ion-exchange separation can be coupled to other detection strategies, including pulsed amperometric detection (PAD), UV/Vis absorbance detection, inductively-coupled plasma mass spectrometric detection (ICPMS), and mass spectrometric detection, providing a wide range of analyte sensitivity and specificity. Ion-exclusion separations expand the range of IC applications to some nonionic analytes (e.g., alcohols) and provide a different selectivity for some analytes that can also be separated by ion exchange. The wide dynamic range of the majority of the IC detection methods makes IC applicable to the quantification of trace contaminants as well as major product components in the same run. IC typically uses dilute acids, alkalis, buffers, or salt solutions as the mobile phase, reducing solvent cost and simplifying disposal logistics. In most cases, the effluent can be disposed of after appropriate neutralization and, when necessary, after dilution with water.

IC is typically performed at or near ambient temperature. As with other forms of HPLC, IC separations are based on varying capacity factors and typically follow the Knox equation. IC is a technique often complementary to reversed-phase and normal-phase HPLC as well as atomic absorption and inductively-coupled plasma techniques in pharmaceutical analysis.

APPARATUS

IC instruments can be either conventional HPLC instruments or instruments specially designed to only perform IC. What type of instrument is chosen depends on the application. Typical components of an IC instrument may include an autosampler, a high pressure pump, a sample loop of suitable size, a guard column, an analytical column, an optional suppressor if conductivity detection is used, a flow-through detector, and a data system (*Figure 1*).

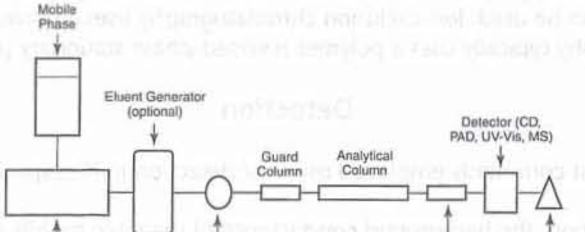

Figure 1. Components of a typical IC system illustrated schematically; CD = conductivity detector, PAD = pulsed amperometric detector, and MS = mass spectrometric detector. [NOTE—Mobile phase is water when the optional *Eluent Generator* is used.]

Because mobile phases generally consist of dilute acids, alkalis, or salt solutions, the components in contact with the mobile phase and the sample are typically made from inert materials, such as polyetheretherketone (PEEK). A conventional HPLC system can be used provided that its components are compatible with the mobile phase and injected sample solutions, though a metal-free system should be used for trace metal analysis. Following suitable preparation, if needed, the sample is introduced via the injection valve. Because IC uses a predominantly ionic mobile phase, a suppressor is typically necessary prior to conductometric detection when high sensitivity is needed, although nonsuppressed conductometric detection has been successfully used in pharmaceutical analysis.

Mobile Phases

Nearly all IC separations require acids or bases diluted in high-purity water, generally with resistivity greater than 18 megohms-cm to prepare the mobile phases. As in every chromatographic method the sensitivity dictates the detection method, and the selectivity dictates the mobile phase and column selection.

For suppressed conductivity detection, the bases and acids used are suppressed to water or weakly dissociated species. If the analytes are anions, the mobile phase bases used as counterions by suppressed conductivity detection are sodium or potassium hydroxide, sodium carbonate, sodium bicarbonate, and less often sodium tetraborate. If the analytes are cations, the mobile phase acids used as counterions by suppressed conductivity detection are methanesulfonic acid, sulfuric acid, and less often hydrochloric acid. When determining low ion concentrations, appropriate trapping technology should be used to purify the mobile phase.

When the detection is based on UV or visible absorbance, then a wide variety of salt solutions may be used to prepare the mobile phase, including phthalic acid and *p*-hydroxybenzoic acid for the determination of anions, and methanesulfonic acid for the determination of cations.

Amperometric detection uses either a strong acid or base solution as the mobile phase, although some methods can use a salt solution near neutral pH or an acid or base solution containing a salt. When using alkaline mobile phases, carbon dioxide/carbonate contamination can be minimized through the use of high purity reagents and storage under nitrogen or helium headspace. Most IC-MS methods use the same mobile phases used for suppressed conductivity, though volatile amines and volatile salt solutions have also been used. IC methods that use an ion-exclusion column use solutions of strong acids such as sulfuric acid as mobile phases. Organic solvents commonly used for HPLC are sometimes used to prepare the mobile phase, typically at NMT 20% concentration. This addition is usually made to either modify selectivity or to enhance solubility of sample components that might otherwise contaminate the stationary phase.

Also, protein molecules may get a different surface charge distribution according to their structure and the pH of the mobile phase. In those cases, the pH, gradient of pH, ionic strength, or a combination of those parameters proved to be useful in the separation.

Stationary Phases

IC separations rely on ion exchange, and therefore stationary phases for IC are anion or cation exchangers, and less commonly phases containing both functionalities. Stationary phases may be silica-based or polymer-based materials supporting ionic functional groups. However, due to the solubility of silica gel in water, particularly at alkaline pHs, the use of silica-based support is limited when the mobile phase is alkaline. In those cases, a polymeric support for IC is useful over an extended pH range. Most of the phases used today are constructed with highly cross-linked polymers, increasing their compatibility with the organic solvents that sometimes are needed to prepare the mobile phase. Polymeric anion-exchange phases for IC are typically constructed from polystyrene/divinylbenzene, ethylvinylbenzene/divinylbenzene, or polyvinyl alcohol polymeric substrate and polymethacrylate supports, with particles sizes ranging from 4 to 15 µm in diameter either nonporous or with pores up to 2000 Å. To provide functionality to the anion exchanger, the anion-exchange groups are attached to the substrate typically in one of two manners, electrostatically or covalently by condensation polymerization (a.k.a. a step-growth polymerization). Cation-exchange phases for IC use the same polymeric substrates as the anion-exchange phases, but because the mobile phases for cation IC are acidic, silica can also be used. Ion-exclusion chromatography uses a porous strong cation-exchange stationary phase, while ion-pair chromatography typically uses a polymer reversed-phase stationary phase.

Detection

Conductivity detection is the most commonly employed mode of detection in IC, especially for suppressed conductivity detection.

In suppressed conductivity detection, the background conductance of the ionic mobile phase is significantly reduced as it flows through the suppression device (suppressor). For example, when diluted sodium hydroxide (10–50 mM) used as the mobile phase in IC of anions flows through the suppressor, it is converted to water (H_2O), which shows very poor conductivity. Analogous reactions occur using a suppressor for cations, where the acidic mobile phase is converted to water, and the analyte cations are converted to highly conducting hydroxide forms (due to higher equivalent conductance of hydroxide ions compared to other anions).

The reduced background conductance and the enhanced signal due to the ionic species result in a significantly enhanced signal-to-noise ratio for the conductometric detection of ions in suppressed IC. This results in reduced baseline noise while increasing the sensitivity and reproducibility of the analysis. Commonly used suppressors can be classified into two categories. In the first type, the reactions occur across an ion-exchange membrane with the regenerant ions furnished by either a chemical or as products of electrolysis of water. In the second type, the suppression reactions occur in a packed bed of high-exchange capacity resin or monolith material, with regeneration either by a chemical or by electrolysis of water.

PULSED/INTEGRATED AMPEROMETRIC DETECTION (PAD/IPAD)

PAD and IPAD are modes of amperometric detection that apply more than one potential to the working electrode. These detection modes are commonly used for the detection of electroactive species, e.g., organic compounds such as carbohydrates, sugar alcohols, amino acids, amines, and organic sulfur species that can be easily oxidized. Analytes are detected by an oxidative desorption process at the surface of an electrode located in the column effluent stream. PAD uses one potential for detection while IPAD uses multiple potentials. The current generated during the fixed time periods these detection potentials are applied is integrated to yield charge. Following detection, a series of potentials are applied for fixed time periods to clean the electrode surface. Unlike conventional direct current amperometry that suffers from electrode surface fouling, this rapidly repeating sequence of potentials for detection and electrode cleaning, referred to as a waveform, allows detection and removal of the products of redox reactions from the working electrode surface.

UV DETECTION

Direct UV detection is used for inorganic and organic ions that absorb UV light, typically at low wavelengths. These include organic acids, bromide, iodide, nitrate, nitrite, thiosulfate, and cyano-metal complexes. Indirect UV detection uses eluents that strongly absorb in the visible or ultraviolet spectral region. A wavelength is selected where the eluent absorbs but the sample ions do not, and then negative peaks proportional to the analyte concentration are detected. In spectral detection after post-column reaction, some analytes are detected after the column effluent is combined with a reagent resulting in the formation of a compound that absorbs light at either a UV or visible wavelength. A classic example is the determination of metal ions, where the metal ions in the column effluent are chelated with 4-(2-pyridylazo)-resorcinol followed by detection at 510 and 530 nm.

MASS SPECTROMETRY (MS)

Typically, analytes are detected after they have first passed through a suppressor to make the resulting effluent compatible with the mass spectrometer. Negative mode electrospray ionization is used for anions while the positive mode is used for cations. The suppressor effluent is sometimes augmented with an organic solvent to improve ionization for increased sensitivity. Certain metal ions can be determined by an ion-exchange separation followed by ICPMS.

SAMPLE PREPARATION

Sample preparation may range from simple sample dissolution or dilution to the proper concentration, often followed by filtration, and in other cases more complex preparations need solid-phase extraction (SPE). If the solution is cloudy and/or contains particulates, then filtration through a syringe filter of 0.45-µm pore size that is suitable for IC is needed. Samples containing a high concentration of ions of the same charge as the target analyte may require a sample pretreatment to selectively remove the high concentration ion.

PROCEDURE

The choice of mobile phase is typically dictated by the choice of column, which in turn is chosen based on the selectivity for the analyte(s) compared to other ions of the same charge known or likely to be present. In situations where the other ions are in high concentration, a column with higher capacity is chosen to prevent column overload. This is especially important for many limit tests, where low concentration of a target analyte is in the presence of a large concentration of another ion of the same charge state. For anion IC, some mobile phases can be prepared from the solid or from commercial concentrates or ready-to-use solutions, e.g., sodium bicarbonate/carbonate. Other mobile phases should be prepared and handled with care, e.g., sodium hydroxide solutions, minimizing air exposure, and prepared from 50% sodium hydroxide solutions. Sodium hydroxide pellets and commercial dilute solutions contain large amounts of carbonate, thus altering the desired composition of the mobile phase. The acid solutions for cation IC are prepared by diluting high-purity concentrated acids. Alternately, carbonate/bicarbonate, hydroxide, and methanesulfonic acid mobile phases can be produced by an eluent generator. Most analyses will require the injection of 5–50 µL of sample solution, but larger volumes may be required for the analysis of low concentration analytes. As in other LC techniques, quantification is made by either internal or external standardization procedures,

where the concentration is calculated by interpolation of the sample response into a calibration curve. IC methods are validated according to the recommendations described in *Validation of Compendial Procedures* ⟨1225⟩.

⟨1066⟩ PHYSICAL ENVIRONMENTS THAT PROMOTE SAFE MEDICATION USE

INTRODUCTION

The physical work environment in healthcare settings is one of the most important potential contributors to medication errors. Data on medication errors reported to the United States Pharmacopeia (USP) Medication Error Reporting Program (2008) show that various physical attributes of the workplace affect human performance on the job. These attributes may help or hinder healthcare providers as they strive to deliver safe, high-quality care to every patient. As noted by the Institute of Medicine (IOM) in its landmark 2001 report, *Quality Chasm*, "Health care today harms too frequently and routinely fails to deliver its potential benefits."

This general chapter describes evidence-based standards for creating and maintaining a physical environment that supports and promotes accurate, safe medication use. The process of safe medication use involves multiple aspects, or stages: procurement, prescribing, transcribing, order entry, preparation, dispensing, and administration, as well as monitoring the medication's effects on the patient. A better understanding of these processes can form a solid foundation for improvement, allowing healthcare providers to reach optimal performance within the medication use system in various practice settings. This general chapter focuses on the characteristics of the physical environment that can promote accurate medication use. Standards are provided when justified by evidence and expert opinion, and a glossary of terms used in this general chapter is provided.

ORGANIZATIONAL SUPPORT

The leadership of a healthcare organization typically determines the operations, policies, and procedures that influence safety. The leaders also establish the workplace design and cultural environment, and they have a powerful influence on the espoused safety culture versus the enacted safety culture. For example, the organizational hierarchy can either enhance safety by encouraging the free flow of information and reinforcing safety behaviors, or can discourage and downplay any safety initiatives. Two reports are available that document issues related to organizational actions and culture as they affect patient safety and care: The IOM's 1999 report, *To Err is Human*, and the IOM's 2006 report, *Preventing Medication Errors*.

Accreditation agencies such as The Joint Commission (TJC), the National Integrated Accreditation for Healthcare Organizations (NIAHO), and the International Organization for Standardization (ISO) emphasize the roles of the organization and leadership in determining safety standards. TJC defines the critical function of leadership and holds the leaders accountable for their systems and processes, whereas NIAHO and ISO call for management review of both quality and safety by the governing body, with the goal of promoting high-quality, safe healthcare.

Organizational influence can be understood as falling somewhere on a continuum ranging from prescriptive controls, in the form of rules and procedures, to discretionary controls, which may rely solely on the individual's experience, according to James Reason (*Human Error*, 1990). Organizational controls that foster a safe environment should include, at a minimum, the reporting, analysis, and intervention plans necessary to shore up defenses against adverse medication events. Systems analysis of errors should include the organizational influences that ultimately affect the workforce at the unit level.

Leadership that is supportive and empowering, at all levels of the organization, should result in optimal physical work environments that help to prevent medication errors and promote accurate medication use. This approach can improve the performance of all persons involved in the medication use process. An organization that aligns leadership goals and resources to a strategic safety focus based on evidence will create safer systems for medication delivery. Although hospitals have been "designed" for patient care, there is a growing body of research that points to evidence-based design (EBD) for safe delivery of care. Other high-risk industries with a strategic safety emphasis share common characteristics, including the use of robust processes for improvement throughout organizations; this can lead to interventions based on scientific data and evidence.

MEDICATION SAFETY ZONES

In the medication use system, the degree of accuracy and safety accomplished is the end result of many interactions between humans, their physical work environments, and the equipment and technology they use. It is important to focus on areas of the work environment where (1) medications are prescribed; (2) orders are entered into a computer or transcribed onto paper; and (3) medications are prepared/compounded, dispensed, and administered. These work areas are commonly referred to as medication safety zones (MSZ). Examples include the work areas around a medication cart on a nursing unit; any location where prescribing decisions are made; the work space of an automated medication dispensing device; a pharmacy where prescriptions are prepared, inspected, and dispensed; and areas in patients' homes where medications are prepared,

consumed, or administered. The patient's bedside in the healthcare facility or home is another important MSZ that presents unique challenges.

The physical environment of any procedure room deserves special attention because of the increased noise levels, variable illumination, and other distractions. In this challenging setting, providers are making critical medication decisions that can influence whether a patient lives or dies. Also, medication safety zones should include the provision for areas that allocate space to incorporate equipment for the safe disposal of sharps (needles and syringes). Overall, in any MSZ, it is important to ensure that appropriate quantities of supplies are maintained; product expiration dates are checked routinely; and temperature-sensitive products are kept in the correct storage conditions.

Principles From Human-Factors Research

The research literature describes several principles that may be useful in planning the physical environment of the MSZ.

IMPORTANCE PRINCIPLE

Within the MSZ, important components should be placed in convenient locations. For example, information systems should be accessible within or near the MSZ so that drug information and patient data (e.g., lab results and vital signs) are readily available. Instructions about equipment function and troubleshooting should be located directly on or near the equipment to provide a quick answer to questions that arise.

FREQUENCY OF USE PRINCIPLE

Supplies and equipment that are used frequently should be easy to find and accessible. This reduces the likelihood that staff will create workarounds, in which suboptimal equipment/supplies are substituted for the recommended items.

FUNCTION PRINCIPLE

Displays, controls, or supplies related to a specific function should be grouped together. For example, syringes, needles, and alcohol swabs could be stored together in one drawer, and IV tubing and connectors that are used to prepare infusions could be placed together on the same shelf.

SEQUENCE OF USE PRINCIPLE

Items should be placed in an order that reflects the sequence of steps needed to perform the task correctly. For example, sterile gloves are in or with sterile dressing kits, and the items are arranged in sequence. This allows personnel to complete tasks quickly and correctly.

Bedside Workstations

Methods for performing workplace analysis are available. One important aspect is the medication administration areas at the bedside (in healthcare facilities or the patient's home), which should follow the same design as the centralized MSZ. Distractions are an even greater challenge at the bedside, and measures should be taken to minimize them whenever possible. Information and supplies should be kept accessible (according to the human-factor principles already discussed) and placed in an uncluttered area with adequate lighting. Sharps containers should be placed within easy reach for the provider, but out of high-traffic areas. Each bedside work station should be standardized in design, so that information and supplies do not need to be brought along when moving from one patient bed to another.

Lean Production

Maintaining an efficient, effective workplace reduces the likelihood of errors. One efficient approach to workspace design uses lean-production techniques to enhance desirable, value-added activities while eliminating undesirable activities that lead to waste during work processes.

Three lean-production techniques are as follows: (1) *Visual Controls*, i.e., keeping work processes and indicators in view so that personnel can see the status of tasks at a glance; (2) *Streamlined Layout*, i.e., optimizing the sequence of work processes through facility design; and (3) *Point-of-Use Storage*, i.e., storing supplies at the location where they will be used whenever possible so that personnel can perform tasks more efficiently.[1]

[1] Sanders MS, McCormick EJ. *Human Factors in Engineering and Design*. New York: McGraw-Hill; 1993.

General Chapters

Simplification and Standardization

Making changes that simplify and standardize the patient-care environment decreases the cognitive load, reducing the likelihood of slips and lapses during routine tasks by minimizing decision and manipulation time. Standardization can be used for facility and room design, medical equipment, and medication-related functions (e.g., medication delivery, storage of patient-specific medications). Ensuring ready access to clinical information that is specific to the patient (or the drug) is essential for all areas in which personnel implement steps in the medication-use process.

Innovative Solutions

Another approach to optimizing the design of the MSZ is to involve personnel who perform the tasks on a routine basis. Workers may suggest innovative solutions to work station problems. To support innovation, it is advisable to incorporate flexibility into the MSZ design.

Forcing Functions

Constraint and forcing functions are an effective means of preventing errors, particularly for high-risk medications and situations. Forcing functions do not always refer to device design. The simplest of these do not require technology. One of the first forcing functions identified in healthcare was the removal of concentrated potassium from hospital units to eliminate the risk of inadvertent preparation of intravenous solutions with concentrated potassium, an error that has caused a small but stable number of deaths over the years.

Other examples would include sealing neuromuscular blockers in an intubation kit which lowers the chance of a paralyzing agent being administered to a patient without having access to ventilation support. Additionally, packaging an enteral product so it is physically unable to connect to an intravenous tubing luer lock connector would avert a wrong route error, even if the nurse was working in low-light conditions and initially misidentified the intended route for the tubing. The availability of medication safety technology is never a substitute for safe medication practices within an MSZ. Reports have warned of errors that result from ignoring or overriding safety checks, such as smart infusion pump drug libraries and alarms.

Work System Elements

CHARACTERISTICS OF INDIVIDUALS

Characteristics of the individual performing the work include his or her experience level, age, visual and hearing acuity, distractibility, and level of attention. Humans vary in their responses to the physical environment. In an ideal scenario, the physical environment could be modified on an individual basis. In that way, the environment could be adapted to match the needs of the current user, thereby optimizing the accuracy of his/her performance. Alternatively, the environment should be as supportive as possible to as wide a range of capabilities as possible.

SOUND AND NOISE

The Environmental Protection Agency (EPA) recommends peak sound levels of 45 dB during the day and 35 dB at night in hospitals. The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB. The International Noise Council recommends maximums of 45 dB during the day and 20 dB at night for acute care areas. Ear protection is required when workers are exposed to sound levels averaging 90 dB.

The standard for sound levels in MSZs is set at 45 dB. This is intended to ensure that critical verbal information can be heard accurately. Healthcare providers should be sensitive to their individual need for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods to enhance audibility and learning, for example, the use of a closed room.

Noise is recognized as a serious health hazard to hospitalized patients, and as a source of interference with effective work performance. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels as a factor contributing to stress for nurses is increasingly being documented. In healthcare facilities, sources of noise can range from overhead paging systems, equipment alarms, heating, ventilation, and air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines. Noise has been cited as one obstacle to the effective performance of nurses. An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB. A study of sound levels during shift changes measured 113 dB.

The following sound-related features may affect accuracy when dispensing medication: predictability of the sound; controllability of the sound; type of task (simple vs. complex); multitasking; and distraction due to noise (which may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks). Out of 58 studies, 7 showed that

noise improved performance, while 29 showed that it impaired performance. Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research. This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes–Dodson law).

Noise and other sensory interference can be reduced by employing activities, tools, and principles developed by human factors and engineering experts; many of these principles are already being used by some healthcare organizations. The effects of these and other design improvements for nursing workspaces on patient outcomes and facility performance are being studied as part of a research project (http://www.healthdesign.org/pebble) sponsored by the Center for Health Design, a nonprofit research and advocacy organization. The project has found decreases in medical errors, as well as reductions in patient transfers, nosocomial infections, patient falls, and medication usage. When permitted by infection control guidelines, reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting) can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. Workers who don't have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

ACTIVITIES AND TASKS PERFORMED

When designing an optimal physical environment that promotes accurate medication use, one should consider the activities and tasks that need to be performed. A poorly designed environment or other adverse conditions in the work area can lead to an unsafe adaptation of the procedures. For example, practitioners who have to listen to the constant barrage of equipment alarms that are not set within appropriate parameters (and are producing too many false alarms) may silence the alarms to reduce the noise. However, this limits the practitioner's ability to monitor the safety of the system. Similar situations occur with visual alarms and computer messages that are often ignored or overridden. In general, activities and tasks should be structured so they can be performed with the least amount of difficulty. If workarounds are detected, they should be investigated as an indication that the procedure or workflow is not suited for the task.

Multi-Tasking

The term "multi-tasking" originated in the computer engineering industry; it is the ability of a person to perform more than one task simultaneously. The human brain is wired differently in every individual; therefore, not everyone can multi-task successfully. The need to multi-task is common in all areas of the healthcare environment, whether on a nursing unit, in a pharmacy, in an operating room, or in a special procedure area. Some people can focus on performing more than one task at a time, whereas others cannot. In the human brain, multi-tasking involves linearly switching back and forth among different activities while actually completing one task at a time.

This can be problematic in healthcare settings, particularly if something unexpected happens in relation to one task while the individual is focused on the other task. These interruptions and distractions can lead to medication errors and other forms of patient harm. Another type of multi-tasking error is related to "mental stacking," that is, when several items are under consideration at the same time. An excessive cognitive workload can lead to unintended consequences, and it is important to note that not all practitioners are able to handle established systems and processes in a particular physical environment.

Pharmacists and nurses are practitioners who typically multi-task. Although most prescriptions are prepared or administered one at a time, this does not mean that multi-tasking cannot occur. The work area should be designed to keep individual prescriptions separate while accommodating multi-tasking. Individuals vary in their abilities to perform sequential tasks versus multi-tasking. Currently there is not enough evidence to evaluate an individual's fitness to perform multi-tasking in healthcare settings, but human factors research in controlled laboratory settings suggests that in the future, it may be possible to assess and augment this skill in healthcare providers.

TOOLS AND TECHNOLOGIES IN THE PHYSICAL ENVIRONMENT

In the healthcare setting, tools and technologies that are used to perform the tasks can also interact with the physical environment to affect the likelihood of medication errors. Therefore, the design of the environment should account for the specific tools and technologies that individuals will use to complete their work. For instance, the physical layout of an operating room should accommodate the size, maneuverability, and required spacing of the many devices used during surgery. Tools and technologies should also meet ergonomic design standards.

Another example is that labels on medications are sometimes too small to display instructions for preparation, dispensing, or administration; when this occurs, alternatives should be considered. One alternative is to include Quick Response (QR) codes on the labels, which allow healthcare personnel with a web-enabled device (such as an iPad, iPhone, or Android-powered device) to access prescriptive information and handle the product correctly. QR codes are two-dimensional bar codes that, when read by an imaging device, bring the user directly to the website containing the needed information.

Additional factors that should be considered include equipment malfunction, downtime, and vibration.

Equipment malfunction: Any equipment that malfunctions, is damaged, or is lost should be reported to the supervisor immediately. In the case of malfunction, a decision should be made either to repair or to replace the item as soon as possible. In many cases, the item can be replaced rapidly, but some pieces of equipment take longer to disassemble and reinstall. Personnel should confirm that the equipment is in good working order before and after each use. A routine program of confirming equipment function should be in place.

Equipment downtime: The term "downtime" refers to periods of time when a system or item of equipment is unavailable. The outage, or downtime duration, is the length of time that the equipment fails to perform its primary function. The concept of downtime is commonly applied to computer networks and servers but can also be used in other environments when discussing equipment failures. These failures can occur for various reasons, including damage; design flaws; procedural error, i.e., improper use by humans; engineering issues, i.e., how the equipment is used and deployed; overload occurring when system resources are stressed beyond capacity; environmental problems with support systems such as heating, ventilation, and air conditioning (HVAC) and power; and scheduled downtime for maintenance, upgrades, or expansion. Service agreements are a common strategy for minimizing downtime and outages. Procedures should be in place to address downtime.

Equipment vibration: Vibration is a mechanical phenomenon in which oscillations occur around an equilibrium point. Although vibration may be desirable, usually it is undesirable, wasting energy and creating unwanted noise. As examples, the vibrational motions of engines, electric motors, or any mechanical device are typically unwanted and may result from imbalances in the rotating parts, uneven friction, imperfect meshing of gear teeth, and other factors. Careful design and proper installation can usually minimize unwanted vibrations.

DISTRACTIONS AND DISTRACTIBILITY INDEX

The healthcare work environment is replete with distractions and interruptions that can adversely affect work performance and lead to medication errors. Architects, engineers, and other professionals who play a role in workplace design have a responsibility to be educated about, and keenly aware of, this issue so they can design safe, ergonomically ideal workspaces. This approach should have a significant beneficial impact by counteracting the negative effects of interruptions and distractions on patient care. In general, MSZs should be located in areas where the potential for distraction and interruption is minimized. Nurses frequently cite distractions and interruptions as contributing to the incidence of medication errors. Distraction from competing tasks is likely to impair performance in several ways, such as sensory/perceptual interference (e.g., the nurse doesn't hear the alarm because a coworker interrupts with a question), cognitive cost of switching tasks (the nurse responds to an alarm more slowly because it takes time to reorient to the alarmed task after a coworker's question), or prospective memory failure (when returning to the task after the interruption, the nurse omits a step because of forgetting where he/she left off).

In addition, interruptions and distractions have been linked to higher rates of prescription dispensing errors in an ambulatory pharmacy. According to the 2008 USP MEDMARX Data Report, distractions continue to play a major role in medication errors, identified as a contributing factor in 45% of all medication errors in hospitals and health systems. However, interruptions and distractions can be prevented or reduced by giving staff the ability to control and manage their exposure to these disturbances. Personnel can be allowed to adjust features of the MSZ to maximize their concentration and attention levels and to optimize their performance. Adjustable features include a work station that is protected from interruptions and distractions, such as a separate medication room, versus a mobile cart with workspace for those that are not adversely affected by distractions. Individuals have different levels of distractibility, and should be sensitive to their own need for a distraction-free work area. Heightened self-awareness of the adverse impact of interruptions and distractions can help minimize problems. Also, workers can be trained in how to avoid interrupting coworkers for non-urgent requests, particularly while their coworkers are performing medication-related tasks. In a pharmacy study, the most frequent source of interruptions was coworkers asking for assistance.

Techniques that may be effective for decreasing interruptions and distractions include visual cues (such as wearing orange safety vests) and physical barriers, such as preparing doses in a medication room. Personnel can also use checklists to focus or refocus their own attention on the task. Scientific research about distractions and interruptions in the physical environment is limited; therefore, additional studies are needed to identify evidence-based corrective actions that will promote safe medication use.

PHYSICAL ENVIRONMENT ELEMENTS

Factors in the physical environment, such as lighting, noise, temperature, layout, and workstation design, interact with human and task-related factors to influence the accuracy of medication use. The remainder of this general chapter focuses on providing guidance on the optimal environment to improve the performance of persons involved in the medication use process.

WORK SYSTEM DESIGN

According to the Systems Engineering Initiative for Patient Safety (SEIPS) model, five work systems—humans, tasks, technology and tools, organization, and environment—interact to affect employee, organization, and patient outcomes. Because

these five work systems are closely interrelated, designers should consider all of the systems. Whenever one work element changes, there will be implications for the other elements. The entire work system needs to be well designed to optimize performance and ensure positive outcomes. Although this general chapter describes all the work systems, it focuses on recommendations for the physical environment.

Evidence-Based Design

Evidence-based design (EBD) is a field of study that draws upon the most reliable research data in planning the design of the workplace environment and equipment. EBD is closely related to the systematic hierarchy of evidence-based practice, and uses a systematic process of evaluating scientific research as the basis for design decisions that enhance human performance and reduce stress in the complex environment of healthcare. EBD intersects and combines the domains of ergonomics, human factors, usability, and cognitive psychology to arrive at the best possible work environment.

EBD principles are available to inform environmental design choices. In 2008, Ulrich and associates published a review of the research literature on evidence-based healthcare design, concluding that "The evidence indicates that well-designed physical settings play an important role in making hospitals safer and more healing for patients and better places for staff to work." For example, major advances such as reduced infection rates have been linked to the design and location of sinks for hand washing, proper ventilation, and suitable materials for surfaces.

Application of EBD principles to equipment design is just as essential as applying these principles to the work environment. The use of EBD for intravenous pumps, feeding tubes, and patient-controlled analgesic devices has been described in recent years. Research continues to support the use of standardized fonts and labels for drug identification, as well as EBD for medication storage areas. In each decision regarding the complex healthcare work environment, stakeholders should draw upon the most substantial research evidence available and use it as a major guiding factor. Objects such as furniture, cabinetry, and fixtures should be selected after careful consideration of EBD goals and research. For example, it is ideal if furniture can be configured to create a sense of privacy and minimize visual and auditory distractions during medication transcription, preparation, dispensing, and administration. Furniture should be adjustable to meet a worker's ergonomic needs. In addition, surface contamination can be reduced by selecting nonporous surface materials with no joints or seams, allowing for ease of cleaning.

CHALLENGES IN THE PHYSICAL ENVIRONMENT

Ordering and Transcribing

Errors in ordering medications or transcribing medication orders are well documented in the research literature. Because errors made during these tasks can directly endanger patient safety, ordering and transcribing should be treated as high-risk activities within the healthcare environment. Manual methods of ordering and transcribing are dependent on human performance. Errors may result when the reader misinterprets handwriting flaws, abbreviations, or decimal points, or when there is frank misreading of handwriting. Faxed orders involve additional concerns, because legibility may be poor after transmission and printing.

Computerized systems for ordering, known as computerized physician order entry (CPOE), and transcription of orders by electronic means are not completely protected from the possibility of error. For example, electronic healthcare records have been cited for failures in usability and visual display, and may lack the customization necessary to order for specific populations, such as pediatric patients. Although the electronic transmission of data is considered more secure from errors of interpretation, the addition of decision support in CPOE has not been established as an evidence-based intervention that assures safety. At all times, applications of technology should undergo the same degree of scrutiny as other methods to prevent medication errors.

Preparation and Compounding

The preparation/compounding work area should be designed, arranged, and maintained to facilitate high-quality compounding that meets the standards of *Pharmaceutical Compounding—Nonsterile Preparations* 〈795〉 and *Pharmaceutical Compounding—Sterile Preparations* 〈797〉. The area for sterile drug preparation should be separate from the area for nonsterile drug preparation (see 〈795〉 and 〈797〉). Traffic in the preparation/compounding area should be kept to a minimum, with only authorized individuals allowed access. Lighting, temperature, and ventilation should be optimal to prevent decomposition of active pharmaceutical ingredients (APIs), excipients, and drug products. These measures can also ensure a workplace where personnel are not distracted by physical discomfort.

Humidity should be monitored and controlled, because drugs tend to degrade in the presence of moisture. Because of its effect on drug stability and integrity, the humidity in compounding and storage areas is second in importance only to temperature (see 〈795〉 and 〈797〉). A hygrometer should be placed on an interior wall, but not near an air-handling return, hot plate, or door entrance/exit, so that it will provide a representative reading of the relative humidity in the compounding area or storage facility.

General Chapters

The materials used for the floor, walls, shelving, cabinets, and ceiling should not retain dust, odors, or residues from the compounding activities. Also, the area should be free of dust-collecting overhangs (e.g., ceiling pipes, hanging light fixtures, and ledges). The actual work area should be level, smooth, impervious (free of cracks and crevices), and non-shedding, and the shelving and cabinets should be easy to clean.

Hazardous drugs should be prepared, stored, and handled by appropriately trained personnel under conditions that protect the healthcare workers and others who may come in contact with these drugs. Disposal of all hazardous drug wastes should comply with the applicable federal and state regulations. Any workers who perform routine custodial waste removal and cleaning activities in the storage and preparation areas for hazardous drugs should be trained in appropriate procedures to protect themselves and prevent contamination of the physical environment. This training should include procedures to be followed in the event of a spill.

For sterile compounding, the primary engineering control (PEC) unit should be placed in a location that will minimize noise and avoid conditions that could adversely affect its operation. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC systems can disrupt the unidirectional airflow in open-faced workbenches. The PEC should be placed out of the traffic flow and in a position that will avoid disruption from the HVAC system and room cross-drafts. In general, all equipment should be selected and installed to create optimal working conditions, with minimal noise and sufficient lighting, so that personnel can perform accurate, high-quality work (see ⟨797⟩).

Dispensing

There are many potential distractions in dispensing areas of pharmacies and other areas where medications are dispensed. These distractions may include ringing telephones and interruptions from patients, other staff, visitors, and others. Distractions should be minimized or eliminated to the greatest extent possible. Although dispensing functions often require multi-tasking, it is optimal that only one prescription or medication order be prepared, processed, and checked at a time. The final check should be done by a pharmacist or an authorized, licensed professional in an area that is free of distractions. Additional points include the following:

- Proper illumination is necessary because the labels on some containers have very small or lightly colored print.
- Noise levels should be kept to a minimum (see *Table 2*), which may require moving noisy equipment to a different room, or at least using a partition for separation.
- Odors in the pharmacy setting should be controlled by using appropriate exhaust systems, which should be maintained properly and monitored periodically. Pharmacies contain a combination of odors resulting from the storage, preparation, and dispensing of the drugs themselves; odors also emanate from other items such as disinfecting agents.
- Temperature should be maintained at appropriate levels for the comfort of pharmacy personnel and for the stability of the pharmaceuticals.
- The pharmacy workspace should be designed to enhance workflow efficiency and to maintain safety throughout the entire dispensing process.

Administration

Some critically important MSZs for nurses include the medication preparation and administration areas. Information should be readily available in a user-friendly format that will facilitate the practitioner's synthesis of facts and data. Access to medication-related information should be efficient, with materials and records available at the proper sites. For example, a nurse may need both drug information and patient-specific data to make a decision about drug administration; therefore, these two sources should be near each other to support fact finding and/or decision making. The various information components within the space should be arranged according to specific principles that decrease distractions when seeking information and making decisions. Nurses also need to be aware of additional factors when administering medications, e.g., illumination, clutter, odor, noise levels, and temperature.

The Home Environment

The home healthcare environment differs from hospitals and other institutional environments in some important ways. For example, clinicians recognize that the homecare setting is the private domain of the patient. Thus the care they provide to each patient should be unique and individualized, based largely on that home setting. There may be situational variables in the patient's home that present risks to the patient and are difficult or impossible for the clinician to eliminate. For instance, hospitals have environmental safety departments to monitor air quality, as well as designers/engineers to ensure that the heights of stair risers are safe, but home healthcare providers are not likely to have the training or resources needed to assess and ameliorate such risks in the patient's home.

Yet the patient and caregiver still can promote a safe home environment by establishing proper storage, organization, and accessibility of medications, as well as adequate lighting in areas where drugs are administered. Several other physical elements also should be assessed to facilitate safe handling and administration of medications. There should be reliable telephone service to the home, an adequate area to prepare and administer medications (e.g., parenteral infusions), and intact, safe elec-

trical outlets for any required equipment. All medicines should be stored in ways that facilitate both medication safety and compliance. The storage locations should be dry and cool. Medications needing refrigeration should be stored in areas where they will not freeze. All medications, whether prescription or over-the-counter, should be kept out of reach of children, pets, or mentally altered/impaired adults.

PHYSICAL ENVIRONMENT FACTORS

Sensory interference resulting from extreme temperatures, noise, poor lighting, glare-producing surfaces, interruptions, or clutter can adversely affect the working memory and job performance of healthcare practitioners. The guidelines described here for the physical environment should be applied to MSZs.

Illumination

Proper illumination levels can improve both accuracy and efficiency of performance. Prescription-filling accuracy improved significantly, from 96.2% to 97.4%, when lighting levels in a busy outpatient pharmacy were increased from 450 to 1460 lux (45–146 fc). One study found that pharmacists who rated lighting levels as at least adequate detected 38% more errors when filling prescriptions. In addition, as visual fatigue increases over a shift, increased light is needed. Pharmacists using task lights to increase illumination had a 10.7% reduction in product verification errors. A study of luminance in homes, offices, and public places found lower levels than recommended for reading, and also found that performance was related to age. Efforts should be made to prevent medication errors caused directly or indirectly by low lighting. For example, one incident report showed that poor lighting contributed to improperly connected patient-controlled analgesia (PCA) administration tubing, causing medication to run onto the floor, resulting in uncontrolled patient pain. Low lighting contributed to difficulty in seeing that the tubing was not connected properly. A study of lighting in a retail pharmacy revealed an error in strength and dosage form as dicyclomine 10-mg capsules were used to fill a prescription for 20-mg tablets. The light level at the shelf where the medications were stored was 220 lux (22 fc).

The recommendations described here take into consideration the need to work quickly and accurately during medication handling, the level of task visibility, and the comfort of personnel. Architects and lighting engineers can consult the Illuminating Engineering Society of North America (IESNA) reference "Lighting for Hospitals and Healthcare Facilities" for details about lighting medication areas. It is important to note that the illuminance levels recommended in the IESNA reference are below those listed in this guidance; the latter are higher because of evidence that lighting levels are inversely related to medication errors. Fluorescent cool white deluxe lamps or compact fluorescent lamps are recommended, because they have a color rendering index of 80 or more and are highly efficient compared to incandescent lamps. Providing the recommended color rendering index can help avoid misidentification of medications.

If illumination levels are below recommendations, task lighting is required in areas where critical visual tasks are performed. If task lighting is not available, then workers can cast shadows on the workspace, further reducing lighting levels. Critical tasks include reading handwritten prescriptions and small print on labels and inspecting medication dosage forms. Because individuals perceive lighting levels differently, adjustable 50-watt high-intensity task lights are recommended for situations where personnel encounter difficult-to-read prescriptions and product labels such as unit-dose package labels. Lighting levels are important for key healthcare providers involved in computer order entry (e.g., physicians or pharmacists), prescription filling, inspection, and patient counseling. Illumination levels for computer order entry areas should be at least 750 lux (75 fc). Higher levels [1000 lux (100 fc)] are recommended when handwritten orders are read.

Lighting should be positioned so there is no glare on the computer monitor that may make it difficult to read the screen accurately. Prescription preparation areas, medication inspection stations (for double-checking), and counseling areas should have illumination levels between 900 and 1500 lux (90–150 fc). These standards are all above the minimum of 200 lux (20 fc) for accurate reading of medication labels set by the American Society for Testing and Materials International (ASTM International). An ASTM International standard includes a legibility test requiring that the name and amount of the drug on the label be legible in 20 fc of light at a distance of about 20 inches (500 mm) by a person with 20/20 unaided or corrected vision. Lighting levels should be increased where the work force has an average age beyond 45 years to optimize legibility (general recommendation for treatment of presbyopia). Healthcare providers should also consider having a magnifying glass available to assist in the careful reading of labels with very small print and in situations where low lighting levels are unavoidable. Pharmacists using a magnification lens along with a task light reduced errors in product verification by 22% compared to a control group.

For nurses, key medication-related tasks that require good lighting include medication order review and medication selection, preparation, and administration. These tasks may take place in one or more locations on the nursing unit, such as the nursing station where patient charts are stored, the medication room, or a patient's room. Transitional lighting is recommended for medication areas on nursing stations and other patient care units to avoid dark and bright spots located next to dimly lit areas. Luminance should enable good color rendering (i.e., color rendering index of 80 or more) to assist with proper medication checking. Task lighting can help achieve appropriate levels of lighting and should be included on mobile medication carts (including those used with bar code medication verification systems). Glare on computer monitors should be controlled by ensuring that there are no light reflections that can wash out the screen and make it difficult to read.

Illumination levels for medication rooms located on nursing units should be at least 1000 lux (100 fc) based on the complexity of the task (e.g., reading small type on medication packages) and the need for accuracy and speed. The higher range of the lighting level should be used when the task requires reading small print. Lighting level recommendations are summarized in *Table 1*. Over time, lighting levels can decrease (e.g., by 25% over a 2-year period), so it is important to clean lighting fixtures routinely in order to maintain recommended luminance levels. Lighting levels should be measured on a quarterly basis. Burned out or flickering bulbs should be replaced promptly.

Table 1. Lighting Level Recommendations for Healthcare Settings

Work Area	Illumination Level	
	Lux	Foot-Candle (fc)
Computer order entry	1000	100
Handwritten order processing	1000	100
Medication filling and checking (pharmacy)	900–1500	90–150
Patient counseling (pharmacy)	900–1500	90–150
Sterile compounding and preparation	1000–1500	100–150
Pharmacy medication storeroom	500	50
Medication preparation area (e.g., nursing station)	1000	100
Medication administration work area (e.g., cart surface, patient room)	1000	100

Proper lighting is also essential at the point of care. Attempting to be patient- and family-friendly may run contrary to the necessary lighting conditions for safe medication administration. If practitioners administer medication at night under low luminance to avoid disturbing the patient or family, this an unsafe practice. Task or spot lighting should be available so practitioners can visually confirm that they have the correct patient (by reading armband or other identification technology), and so the medication and administration site is not compromised. Compact fluorescent lamps take time to come to the correct lighting level. Therefore, no critical tasks should be performed until the light is working at its rated level.

Sound and Noise Recommendations

For hospitals, the Environmental Protection Agency (EPA) recommends peak sound levels of 45 decibels (dB) during the day and 35 dB at night in hospitals. The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB. The International Noise Council recommends limits of 45 dB during the day and 20 dB at night for acute care areas. Ear protection is required when workers are exposed to sound levels averaging 90 dB. Sound level recommendations are shown in *Table 2*.

Table 2. Peak Sound Level Recommendations

Work Area	Source[a]	Sound Level (dB)
Hospitals (daytime)	EPA	45
Hospitals (nighttime)	EPA	35
Patient room	WHO	35
Acute care areas (daytime)	INC	45
Acute care areas (nighttime)	INC	20
Ear protection required	INC	90 (average)
Medication safety zones	—	50

[a] EPA, Environmental Protection Agency; WHO, World Health Organization; INC, International Noise Council.

The standard for sound levels in MSZs is set at 45 dB, which is intended to ensure that critical verbal information can be heard accurately. However, this will be exceeded in many typical pharmacy dispensing and compounding areas. Healthcare providers should be sensitive to their individual needs for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods (e.g., use of a closed room) to enhance audibility, learning, and privacy.

Excessive noise is recognized as a serious health hazard. Its effect on hospitalized patients and its possible interference with effective work performance by clinicians should be considered. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels are being documented more often as a contributor to stress for nurses. In healthcare facilities, sources of noise can include overhead paging systems, equipment alarms, heating, ventilation, air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines. Noise has been cited as one obstacle to the effective performance of nurses. An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other

hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB. A study of sound levels during shift changes measured 113 dB.

Accuracy when dispensing medication can be affected by specific characteristics of the sound/noise, e.g., whether it is predictable and whether it can be controlled. These factors may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks. Out of 58 studies, 7 showed that noise improved performance, while 29 showed that it impaired performance. Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research. This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes–Dodson law).

The adverse effects of noise and other sensory interference can be reduced by using activities, tools, and principles developed by human factors and engineering experts. Many of these principles and tools are already being used by some healthcare organizations. Researchers are studying these and other design characteristics of nursing workspaces to assess their effects on patient outcomes and facility performance; one study is sponsored by the Center for Health Design, a nonprofit research and advocacy organization, and a network of 11 healthcare providers (http://www.healthdesign.org/pebble). The data have shown reductions in medical errors, patient transfers, nosocomial infections, patient falls, and medication usage. Reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting), when permitted by infection control guidelines, can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. In addition, workers who do not have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

Odor

Areas where pharmaceuticals are stored or manipulated tend to have an odor that is characteristic of the respective drugs. These odors often can be minimized by using appropriate air handling/exhaust systems. Indoor environmental quality (IEQ) can be related to chemicals and other sources. The odors found in almost any working environment may result from caulks, sealants, coatings, adhesives, paints, varnishes, stains, wall coverings, cleaning agents, fuels and combustion products, carpeting, vinyl flooring, fabric materials, air fresheners, and other scented products, as well as personal products of employees (e.g., perfumes, shampoos, and others). If these odors are not controlled, IEQ problems can arise, especially if the building's ventilation system is improperly designed and/or poorly maintained. IEQ can be improved by installing appropriate exhaust systems to remove the odors emanating from drug products. This may also include "snorkel exhaust" facilities close to where the drug products are manipulated, as well as cabinet-type exhaust hoods. Other measures that may be helpful include establishing perfume-free zones, prohibiting smoking, and not allowing food storage or eating in the immediate area. The practice of introducing pleasant odors in the workplace may or may not be beneficial, but managing the response to odors and irritants is critical to maintaining the health and well-being of workers.

Temperature Controls and Humidity

Temperature control is important for drug stability and for the comfort of personnel. In a room where equipment is present (e.g., laminar air flow hoods), heat tends to be generated, and therefore, appropriate temperature control measures should be designed and implemented.

In some situations such as compounding, it is important to control humidity and thereby minimize static electricity in powders, packaging, and other dosage forms (see ⟨795⟩ and ⟨797⟩). Humidity control is also important for related technologies, such as computers, tablets, and automated dispensing machines. The core temperature of the central processing unit in computers should be between 10° and 32° C, and the ideal humidity index is between 30% and 50%.

Physical Design and Organization of Workspace Assessment

The design of the work environment influences the ability of providers to effectively use information and accurately perform tasks. The height of counters, height of shelving for storage of supplies, and lighting changes in lower drawers and cabinets that decrease visibility of products can contribute to errors if improperly adjusted. The effective integration of adjustable fixtures and appropriate counter heights, with the use of mobile carts, can improve efficiency as well as safety. Work counter clutter or lack of sufficient space to perform key tasks is typically an indicator of disorganization and poor planning. One study found that more dispensing errors occurred when medication storage containers were placed on shelves in a cluttered fashion (<1 inch between distinct drugs). When choosing appropriate furniture, an evidence-based-design furniture checklist may be used to make informed investment decisions and improve healthcare outcomes (see *Table 3* for a complete checklist).

General Chapters

Table 3. Evidence-Based Design Checklist

Findings	Evidence-Based Design Goals and Features[a]
	1. Reduce surface contamination linked to healthcare associated infections
	a. Surfaces are easily cleaned, with no surface joints or seams.
	b. Materials for upholstery are impervious (nonporous).
	c. Surfaces are nonporous and smooth.
	2. Reduce patient falls and associated injuries
	a. Chair seat height is adjustable.
	b. Chair has armrests.
	c. Space beneath the chair supports foot position changes.
	d. Chair seat posterior tilt angle and seat back recline facilitate patient egress.
	e. Chairs are sturdy, stable, and cannot be easily tipped over.
	f. Rolling furniture includes locking rollers or casters.
	g. Chairs have no sharp or hard edges that can injure patients who fall or trip.
	3. Decrease medication errors
	a. Lighting fixtures should provide 90–150 foot-candle illumination and an adjustable 50-watt high intensity task lamp for furniture with built-in lighting that is used in a medication safety zone.
	b. Furniture is configurable to create a sense of privacy to minimize visual distractions and interruptions from sound and noise during medication transcription, preparation, dispensing, and administration activities.
	4. Improve communication and social support for patients and family members
	a. Furniture can be configured into small flexible groupings that are easily adjusted to accommodate varying numbers of individuals in a variety of healthcare settings.
	b. Wide-sized and age variations are supported.
	c. Acoustic and visual patient privacy are supported.
	5. Decrease patient, family member, and staff stress and fatigue
	a. Materials suggest a link to nature.
	b. Appearance is attractive and noninstitutional.
	c. Furniture is tested for safe and comfortable use by all, including morbidly-obese individuals.
	6. Improve staff effectiveness, efficiency, and communication
	a. Furniture is easily adjustable to individual worker's ergonomic needs.
	b. Design enables care coordination and information sharing.
	c. Materials are sound absorbing.
	7. Improve environmental safety
	a. Materials do not contain volatile organic compounds (VOCs), such as formaldehyde and benzene.
	8. Represent the best investment
	a. Reflect and reinforce the organizational mission, strategic goals, and brand.
	b. Integrate new with existing furniture and objects for facility renovations projects.
	c. Pieces can be flexibly reconfigured and moved to support changing and emerging missions.
	d. Provide casters or glides to reduce floor damage.
	e. Check that there are no protuberances that may damage walls; check chair rail heights.
	f. Manufacturer provides results of safety and durability testing.
	g. Manufacturer describes the specific evidence that has been used to design the product.
	h. Manufacturer includes a warranty appropriate to use, such as furniture used all day, every day.
	i. Replacement parts are available.
	j. Repair can be done in the healthcare facility.
	k. Manufacturer or local dealer can assist with furniture repair and refurbishing.
	l. Environmental services/housekeeping staff can easily maintain furniture.
	m. A Group Purchasing Organization (GPO) can be used when purchasing furniture.

Findings Scale:
Present (+), Absent (–), More Information Needed (?), Not Applicable (N/A).
[a] Malone EB, Dellinger BA. *Furniture Design Features and Healthcare Outcomes.* The Center for Health Design; 2011 (see https://www.healthdesign.org/chd/research/furniture-design-features-and-healthcare-outcomes).

General Chapters

METHODS FOR ASSESSING THE PHYSICAL ENVIRONMENT

Illumination

An illuminance meter, also referred to as a light level meter or photometer, is an instrument that consists of a photodetector (with a digital or analog display) that measures illuminance in lux or foot-candles (fc). Lighting levels should be evaluated in all MSZs using point illuminance measurements. To do this, the photodetector should be placed in the area where the critical medication task is performed (e.g., medication inspection at a work counter), with the worker standing in a normal working position when the measurement is taken. In medication storage areas, measurements should include light levels at the top, middle, and bottom shelves, because light levels depend on the distance from the lighting source. Photometers are commercially available, or management engineers may be able to provide them, and they should be recalibrated annually.

Illumination levels also can be measured using a smart phone "Light Meter" application (app). These apps are easy to use and inexpensive. They are simply downloaded to a Smartphone, and after clicking on the app, the camera function is pointed to a lighted area/surface and the on/off button is pressed. The device takes measurements and also provides a description of the activities for which those light levels are appropriate. The apps can be calibrated and the sensitivity adjusted, with readouts in either lux or fc.

Sound Levels

Sound level meters capable of reading from 30 to 130 decibels on the A scale (dBA) should be used to measure sound levels. The A scale is commonly used when measuring decibels because it most closely represents what the human ear hears in terms of loudness. The meters should be calibrated prior to each use. Measurements are taken while standing in a working position, using the instructions provided in the manual for the specific sound meter. Type 1 or Type 2 meters have acceptable levels of accuracy. Sound levels also can be measured easily and inexpensively by using a Smartphone app. The app is downloaded to a Smartphone, and the readout options are selected. The measurement is activated, and it continues to provide a complete readout of frequency (*x*-axis) and decibel sound pressure level (*y*-axis) in real time.

Odor Detection

Odors can be detected by individuals, but this approach is very subjective. Some odors indicate the presence of a specific chemical entity or moiety. However, appropriate measuring devices for use in a work environment may not be available or practical. Consequently, it may be necessary to select a team of individuals to categorize what is acceptable and what is not acceptable in terms of odors in the work environment. Appropriate HVAC systems may play a role in reducing odors.

Temperature/Humidity Measurement

Temperature is typically measured with digital thermometers, and humidity is measured with hygrometers. In both cases, a recording instrument (e.g., recording thermometer) offers the advantage of documentation.

SUMMARY

As early as the 19th century, Florence Nightingale identified the importance of physical environments for patient healing, but only recently was it established that the physical environment can support the performance of critical tasks by healthcare personnel to protect the patient from iatrogenic harm. Currently, medication errors are recognized as complex, multifactorial, and system dependent, and full scientific understanding of error prevention has proven elusive. However, Nightingale's counsel, "above all, do no harm," compels us to use the most up-to-date evidence for safe practices in healthcare to prevent errors and protect the patient. Aspects and considerations of the physical work environment discussed in this general chapter are not exhaustive, but can serve as a stimulus for the assessment of existing work spaces and for consideration of the latest evidence from human factors science in the design of new facilities. The remaining constituent not discussed in this general chapter is the healthcare provider working in arduous conditions who may, with the best intent, contribute to patient harm through inadvertent actions in substandard conditions. The physical work environment should be structured to support and promote safe delivery of care and prevent harm to patients while also bolstering the practice of healthcare by clinicians.

GLOSSARY OF TERMS

Administering: The preparation, directly prior to use, of a pharmacologic or other therapeutic agent for ingestion, injection, insertion, or application.

Color rendering index (CRI): An expression of how a light source affects the color appearance of objects or humans, compared to how they would appear under a reference light source.

Compounding: The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice.

Constraint: A rule stating under what conditions an action is allowed or prohibited. Constraints are used in designing procedures or tools to prevent unsafe practices.

Crowding: A condition that occurs when multiple workers use the same workspace, adversely affecting the amount of space available for each person and also increasing the negative factors of distractions, interruptions, and noise.

Decibel: A unit used to measure the intensity of a sound by comparing it with a standard level on a logarithmic scale, thereby indicating the degree of loudness. The A scale is commonly used when measuring decibels, because it most closely represents what the human ear perceives in terms of loudness.

Dispensing: The act of providing a medication or prescription order; to fill a prescription.

Distraction: An external stimulus that occurs when a person is engaged in a task or activity that causes a cognitive or emotional disturbance, but does not result in the discontinuation of the activity, such as a telephone ringing or question from a coworker.

Ergonomic design: Arrangement of a workspace to accommodate each individual's capacities and limitations, allowing them to work safely and efficiently. This includes an optimum ambient environment and adjustable furniture.

Forcing function: An aspect of a design that prevents a target action from being performed, or that allows its performance only if another specific action is performed first.

Human factors: The scientific discipline concerned with interactions among humans and other elements of a system, and the profession that applies the theory, principles, data, and methods to design systems that optimize human well-being and overall system performance.
Illumination level: The quantity of light energy reaching an area as measured (in lux or foot-candles) by a photometer with an illuminance sensor; this indicates brightness. A lux is a unit of illuminance, measured in lumens per square meter. A foot-candle (fc) is lumens/square foot, and is also commonly measured by light meters. The term candela replaced fc as the International System (SI) measure of luminous intensity and represents 1 lumen/steradium (lm/st).
Interruption: The cessation of productive activity before a task is completed, caused by an externally imposed stimulus.
Lean production: High-quality work output achieved while eliminating waste and decreasing resources used, time spent, and errors.
Medication safety zone: A critical area where medications are prescribed; orders are entered into a computer or transcribed onto paper documents; or medications are prepared/compounded or administered. The characteristics of an optimal physical environment for accurate medication use will apply to MSZs.
Multi-tasking: The ability of human beings to perform multiple tasks simultaneously.
Noise and sound: Noise is defined as an auditory stimulus that bears no informational relationship to the task at hand. Sound is a change in volume that has some informational relationship to the task at hand. A quiet work environment is defined as an area where noise is absent and the worker is free from disturbance.
Override: To neutralize or counteract the action of an automatic control.
Photometer: An instrument for measuring photometric quantities such as illuminance.
Physical environment: The surroundings that can affect one or more human senses.
Workaround: A plan or method used to circumvent a problem (as in computer software) without eliminating it.
Working conditions: A set of factors that include the physical environment, workforce staffing, workflow design, personal/social factors, and organizational characteristics.

⟨1072⟩ DISINFECTANTS AND ANTISEPTICS

INTRODUCTION

A sound cleaning and sanitization program is needed for controlled environments used in the manufacture of Pharmacopeial articles to prevent the microbial contamination of these articles. Sterile drug products may be contaminated via their pharmaceutical ingredients, process water, packaging components, manufacturing environment, processing equipment, and manufacturing operators. Current Good Manufacturing Practices (cGMPs) emphasize the size, design, construction, and location of buildings and construction materials, and the appropriate material flow to facilitate cleaning, maintenance, and proper operations for the manufacture of drug products. When disinfectants are used in a manufacturing environment, care should be taken to prevent the drug product from becoming contaminated with chemical disinfectants as a result of the inherent toxicity of the disinfectants. The requirements for aseptic processing include readily cleanable floors, walls, and ceilings that have smooth and nonporous surfaces; particulate, temperature, and humidity controls; and cleaning and disinfecting procedures to produce and maintain aseptic conditions. The cleaning and sanitization program should achieve specified cleanliness standards, control microbial contamination of products, and be designed to prevent the chemical contamination of pharmaceutical ingredients, product-contact surfaces and/or equipment, packaging materials, and ultimately the drug products. These principles also apply to nonsterile dosage forms where the microbial contamination is controlled by the selection of appropriate pharmaceutical ingredients, utilities, manufacturing environments, sound equipment cleaning procedures, products especially formulated to control water activity, inclusion of suitable preservatives, and product packaging design.

In addition to disinfectants, antiseptics are used to decontaminate human skin and exposed tissue and may be used by personnel prior to entering the manufacturing area. Chemical sterilants may be used to decontaminate surfaces in manufacturing and sterility testing areas. Furthermore, sterilants may be used for the sterilization of Pharmacopeial articles, and UV irradiation may be used as a surface sanitizer.

This general information chapter will discuss the selection of suitable chemical disinfectants and antiseptics; the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; the application of disinfectants in the sterile pharmaceutical manufacturing area; and regulation and safety considerations. Biofilm formation and its relationship to disinfectants are outside the scope of this chapter. Additional information not covered in the chapter may be obtained from standard texts on disinfectants and antiseptics.[1]

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[1] Ascenzi, J.M., Ed. *Handbook of Disinfectants and Antiseptics*, 5th ed.; Marcel Dekker: New York, 1995; Block, S.S., Ed. *Disinfection, Sterilization, and Preservation*, 5th ed.; Lippincott Williams & Wilkins Publishers: Philadelphia, 2000. Russell, A.D.; Hugo, W.B.; Ayliffe, G.A.J., Eds. *Principles and Practices of Disinfection, Preservation and Sterilization*, 3rd ed.; Blackwell Science Inc.: London, 1999.

DEFINITIONS

Antiseptic—An agent that inhibits or destroys microorganisms on living tissue including skin, oral cavities, and open wounds.

Chemical Disinfectant—A chemical agent used on inanimate surfaces and objects to destroy infectious fungi, viruses, and bacteria, but not necessarily their spores. Sporicidal and antiviral agents may be considered a special class of disinfectants. Disinfectants are often categorized as high-level, intermediate-level, and low-level by medically oriented groups based upon their efficacy against various microorganisms.

Cleaning Agent—An agent for the removal from facility and equipment surfaces of product residues that may inactivate sanitizing agents or harbor microorganisms.

Decontamination—The removal of microorganisms by disinfection or sterilization.

Disinfectant—A chemical or physical agent that destroys or removes vegetative forms of harmful microorganisms when applied to a surface.

Sanitizing Agent—An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

Sporicidal Agent—An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

Sterilant—An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores. Sterilants are liquid or vapor-phase agents.

Microorganisms differ greatly in their resistance to disinfection agents. The order of resistance of clinically significant microorganisms to chemical disinfectants from most to least resistant is listed in *Table 1*.

Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)

Type of Microorganisms	Examples
Bacterial spores	*Bacillus subtilis* and *Clostridium sporogenes*
Mycobacteria	*Mycobacterium tuberculosis*
Nonlipid-coated viruses	Poliovirus and rhinovirus
Fungal spores and vegetative molds and yeast	*Trichophyton*, *Cryptococcus*, and *Candida* spp.
Vegetative bacteria	*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella* spp.
Lipid-coated viruses	Herpes simplex virus, hepatitis B virus, and human immunodeficiency virus

CLASSIFICATION OF DISINFECTANTS

Chemical disinfectants are classified by their chemical type. This includes aldehydes, alcohols, halogens, peroxides, quaternary ammonium compounds, and phenolic compounds (see *Table 2*).

Table 2. General Classification of Antiseptics, Disinfectants, and Sporicidal Agents

Chemical Entity	Classification	Example
Aldehydes	Sporicidal agent	2% Glutaraldehyde
Alcohols	General purpose disinfectant, antiseptic, antiviral agent	70% Isopropyl alcohol, 70% alcohol
Chlorine and sodium hypochlorite	Sporicidal agent	0.5% Sodium hypochlorite
Phenolics	General purpose disinfectant	500 µg per g Chlorocresol, 500 µg per g chloroxylenol
Ozone	Sporicidal agent	8% Gas by weight
Hydrogen peroxide	Vapor phase sterilant, liquid sporicidal agent, antiseptic	4 µg per g H_2O_2 vapor, 10%–25% solution, 3% solution
Substituted diguanides	Antiseptic agent	0.5% Chlorhexidine gluconate
Peracetic acid	Liquid sterilant, vapor phase sterilant	0.2% Peracetic acid, 1 µg per g peracetic acid
Ethylene oxide	Vapor-phase sterilant	600 µg per g Ethylene oxide
Quaternary ammonium compounds	General purpose disinfectant, antiseptic	Concentration dependent on application, Benzalkonium chloride
β -Propiolactone	Sporicidal agent	100 µg per g β -Propiolactone

The effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) registers chemical disinfectants marketed in the United States and requires manufacturers to supply product information on the use dilution, type of microorganisms killed, and

the necessary contact time. Certain liquid chemical sterilizers intended for use on critical or semicritical medical devices are defined and regulated by the U.S. Food and Drug Administration (FDA).

SELECTION OF AN ANTISEPTIC FOR HAND AND SURGICAL SITE DISINFECTION

Hands and surgical sites are disinfected in a hospital setting to reduce the resident flora and to remove transient flora (e.g., *Streptococcus pyogenes*) and methicillin-resistant *S. aureus* and *P. aeruginosa* that have been implicated in hospital-associated infection. Use of antiseptics to disinfect hands has been shown to be more effective than soap and water in reducing the counts of bacteria on the skin; repeated antiseptic use further reduces these counts. These principles may be applied to cleanroom operators in the pharmaceutical industry.

Common antiseptics include 4% chlorhexidine, 10% povidone–iodine, 3% hexachlorophene, 70% isopropyl alcohol, and 0.5% chlorhexidine in 95% alcohol.

SELECTION OF A DISINFECTANT FOR USE IN A PHARMACEUTICAL MANUFACTURING ENVIRONMENT

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following points should be considered: the number and types of microorganisms to be controlled; the spectrum of activity of commercially available disinfectants; the claims as a sterilant; the disinfectant or sanitizer supported by the EPA registrations; the concentration, application method, and contact time of the disinfectant; the nature of the surface material being disinfected and its compatibility with the disinfectant; the amount of organic compounds on the surface that may inactivate a disinfectant; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment with repeated application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning agents and other disinfectants; the planned disinfectant rotation; and the steps that need to be taken to avoid the contamination of pharmaceutical products by a disinfectant.[2]

General Chapters

THEORETICAL DISCUSSION OF DISINFECTANT ACTIVITY

Plots of the log of the number of microorganisms per mL surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time.

The rate constant, K, for the disinfection process can be calculated by the formula:

$$(1/t)(\log N_0/N)$$

in which t is the time, in minutes, for the microbial count to be reduced from N_0 to N; N_0 is the initial number of organisms, in cfu per mL; and N is the final number, in cfu per mL, of organisms.

As with a first-order chemical reaction, the same concentration of disinfectant reduces the number of organisms more rapidly at elevated temperatures. This can be expressed as a temperature, T, coefficient per 10° rise in temperature, Q_{10}, calculated by the formula:

$$\text{Time to decontamination at T°/Time to decontamination at T}$$

in which T is T° – 10.

Further evidence that a first-order reaction is an inadequate description of disinfection is that the Q_{10} values for chemical and enzyme reactions are 2 to 3, while the common disinfectants phenol and alcohol have a Q_{10} of 4 and 45, respectively.

Critical to the successful employment of disinfectants is an understanding of the effect of disinfectant concentration on microbial reduction. A plot of the log of the time to reduce the microbial population in a standard inoculum to zero against the log of the disinfectant concentration is a straight line with the slope of the line termed the concentration exponent, n. The relationship can be expressed as follows:

$$n = (\text{log of the kill time at concentration } C_2) - (\text{log of the kill time at concentration } C_1)/(\log C_1 - \log C_2)$$

in which C_1 and C_2 are the higher and lower disinfectant concentrations, respectively.

The wide differences in concentration exponents, n, have practical consequences in picking the use dilution of different disinfectants and in using dilution to neutralize a disinfectant in disinfectant-effectiveness testing and routine microbial monitoring of the manufacturing environment. For example, mercuric chloride has a concentration exponent of 1, so a 3-fold dilution will reduce the disinfectant activity by 3^1 (or by one-third), while phenol with a concentration exponent of 6 will have a 3^6 (or a 729-fold) reduction in disinfectant activity. Disinfectants with a larger concentration exponent or dilution coefficient rapidly lose activity when diluted. The concentration exponents for some disinfectants are listed in *Table 3*.

[2] Denny, V.F.; Marsik, F.J. Current Practices in the Use of Disinfectants within the Pharmaceutical Industry. *PDA J. of Pharmaceutical Sci. and Tech.*, 1997, *51*, (6), 227–228.

Table 3. Concentration Exponents of Common Antiseptics, Disinfectants, and Sterilants

Disinfectant	Concentration Exponents
Hydrogen peroxide	0.5
Sodium hypochlorite	0.5
Mercuric chloride	1
Chlorhexidine	2
Formaldehyde	1
Alcohol	9
Phenol	6
Quaternary ammonium compounds	0.8 to 2.5
Aliphatic alcohols	6.0 to 12.7
Phenolic compounds	4 to 9.9

Another important consideration may be the pH of the disinfectant. Many disinfectants are more active in the ionized form, while others are more active in the nonionized form. The degree of ionization will depend on the pK_a of the agent and the pH of the disinfection environment. For example, phenol, with a pK_a of 10, will be more effective at a pH below 7 where it is nonionized.

MECHANISM OF DISINFECTANT ACTIVITY

Table 4 lists the sites and modes of action of some representative disinfectants.

Table 4. Mechanism of Disinfectant Activity Against Microbial Cells

Target	Disinfectant
Cell wall	Formaldehyde, hypochlorite, and glutaraldehyde
Cytoplasmic membrane, action on membrane potential	Anilides and hexachlorophene
Membrane enzymes, action on electron-transport chain	Hexachlorophene
Action on ATP	Chlorhexidine and ethylene oxide
Action on enzymes with –SH groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, and iodine
Action on general membrane permeability	Alcohols, chlorhexidine, and quaternary ammonium compounds
Cell contents, general coagulation	Chlorhexidine, aldehydes, and quaternary ammonium compounds
Ribosomes	Hydrogen peroxide
Nucleic acids	Hypochlorites
Thiol groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, and hypochlorite
Amino groups	Ethylene oxide, glutaraldehyde, and hypochlorite
General oxidation	Hypochlorite

MICROBIAL RESISTANCE TO DISINFECTANTS

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less likely to occur at significant levels, as disinfectants are more powerful biocidal agents than antibiotics. In addition, they are normally applied in high concentrations against low populations of microorganisms usually not growing actively, so the selective pressure for the development of resistance is less profound. However, the most frequently isolated microorganisms from an environmental monitoring program may be periodically subjected to use-dilution testing with the agents used in the disinfection program to confirm their susceptibility, as there are real differences among different species in resistance to the lethal effects of different sanitizers.

DISINFECTANT CHALLENGE TESTING

Under FIFRA, the EPA requires companies that register public health antimicrobial pesticide products including disinfectants, sanitization agents, sporicidal agents, and sterilants to ensure the safety and effectiveness of their products before they are sold or distributed. Companies registering these products must address the chemical composition of their product, include toxicology data to document that their product is safe if used as directed on the label, include efficacy data to document their claims of effectiveness against specific organisms and to support the directions for use provided in the labeling, and provide labeling that reflects the required elements for safe and effective use. While these directions provide valuable information, they may not be helpful in terms of the products' use as disinfectants in a manufacturing environment.

In the United States, the official disinfectant testing methods are published by AOAC International[3] and include the Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporicidal Carrier Test. A scientific study submitted for EPA review in support of disinfectant registration must be conducted at a laboratory facility that follows the Good Laboratory Practices (GLP) regulations (21 CFR 58). To demonstrate the efficacy of a disinfectant within a pharmaceutical manufacturing environment, it may be deemed necessary to conduct the following tests: (1) use-dilution tests (screening disinfectants for their efficacy at various concentrations and contact times against a wide range of standard test organisms and environmental isolates); (2) surface challenge tests (using standard test microorganisms and microorganisms that are typical environmental isolates, applying disinfectants to surfaces at the selected use concentration with a specified contact time, and determining the log reduction of the challenge microorganisms); and (3) a statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant. This is considered necessary because critical process steps like disinfection of aseptic processing areas, as required by GMP regulations, need to be validated, and the EPA registration requirements do not address how disinfectants are used in the pharmaceutical, biotechnological, and medical device industries. For the surface challenge tests, the test organisms are enumerated using swabs, surface rinse, or contact plate methods. Neutralizers that inactivate the disinfectants should be included in either the diluent or microbiological media used for microbial enumeration or both. Information on disinfectant neutralization may be found in *Validation of Microbial Recovery from Pharmacopeial Articles* 〈1227〉.

The disinfectant efficacy test must have realistic acceptance criteria. In practice, sufficient organisms need to be inoculated on a 2-inch × 2-inch square of the surface being decontaminated, i.e., a coupon, to demonstrate at least a 2 (for bacterial spores) to 3 (for vegetative bacteria) log reduction during a predetermined contact time (i.e., 10 minutes over and above the recovery observed with a control disinfectant application). The efficacy of the neutralizers and their ability to recover inoculated microorganisms from the material should be demonstrated during the use-dilution or surface-challenge studies. Points to remember are that disinfectants are less effective against the higher numbers of microorganisms used in laboratory challenge tests than they are against the numbers that are found in clean rooms (see *Microbiological Control and Monitoring of Aseptic Processing Environments* 〈1116〉); that inocula from the log growth phase that are typically employed in laboratory tests are more resistant, with the exception of spores formed during the static phase, than those from a static or dying culture or stressed organisms in the environment; and that microorganisms may be physically removed during actual disinfectant application in the manufacturing area.

Although not all inclusive, typical challenge organisms that may be employed are listed in *Table 5*.

Table 5. Typical Challenge Organisms

AOAC Challenge Organisms	Typical Environmental Isolates
Bactericide: *E. coli*, ATCC 11229; *S. aureus*, ATCC 6538; *P. aeruginosa*, ATCC 15442	Bactericide: *M. luteus, S. epidermidis, Coynebacterium jeikeium, P. vesicularis*
Fungicide: *C. albicans*, ATCC 10231 or 2091; *Penicillium chrysogenum*, ATCC 11709; *A. brasiliensis*, ATCC 16404	Fungicide: *P. chrysogenum, A. brasiliensis*
Sporicide: *B. subtilis*, ATCC 19659	Sporicide: *B. sphaericus, B. thuringiensis*

Because a wide range of different materials of construction are used in clean rooms and other controlled areas, each material needs to be evaluated separately to validate the efficacy of a given disinfectant. *Table 6* contains a list of common materials used in clean room construction.

Table 6. Typical Surfaces to be Decontaminated by Disinfectants in a Pharmaceutical Manufacturing Area

Material	Application
Stainless steel 304L and 316L grades	Work surfaces, filling equipment, and tanks
Glass	Windows and vessels
Plastic, vinyl	Curtains
Plastic, polycarbonate	Insulation coating
Lexan® (plexiglass)	Shields
Epoxyl-coated gypsum	Walls and ceilings
Fiberglass-reinforced plastic	Wall paneling
Tyvek®	Equipment wraps
Terrazzo tiles	Floors

DISINFECTANTS IN A CLEANING AND SANITIZATION PROGRAM

The selection of suitable disinfectants and the verification of their effectiveness in surface challenge testing is critical in the development of a cleaning and sanitization program.

[3] AOAC International Official Methods of Analysis, 15th, 16th, and 17th editions. Arlington, VA.

General Chapters

Issues associated with the successful implementation of such a program are the development of written procedures, staff training, decisions on disinfectant rotation, institution of application methods and contact times, environmental monitoring to demonstrate efficacy, and personnel safety.

The cGMP 21 CFR 211.67, *Equipment Cleaning and Maintenance*, details the requirements for written procedures for cleaning, maintenance, and sanitization of pharmaceutical manufacturing equipment. These procedures should address the assignment of responsibility, establishment of schedules, details of cleaning operations, protection of clean equipment prior to use, inspection for cleanliness immediately prior to use, and maintenance of cleaning and sanitization records.

Staff involved in disinfection require training in microbiology, industry practices for cleaning and sanitization, safe handling of concentrated disinfectants, the preparation and disposal of disinfectants, and appropriate application methods. It should be emphasized that the preparation of the correct dilutions is critical because many disinfectant failures can be attributed to use of disinfectant solutions that are too dilute. Typically disinfectants used in aseptic processing and filling areas are diluted with Sterile Purified Water, and are prepared aseptically. Alternately, the disinfectant may be diluted with Purified Water, and then sterile filtered to eliminate microorganisms that may potentially persist in a disinfectant. Diluted disinfectants must have an assigned expiration dating justified by effectiveness studies.

The rotation of an effective disinfectant with a sporicide is encouraged. It is prudent to augment the daily use of a bactericidal disinfectant with weekly (or monthly) use of a sporicidal agent. The daily application of sporicidal agents is not generally favored because of their tendency to corrode equipment and because of the potential safety issues with chronic operator exposure. Other disinfection rotation schemes may be supported on the basis of a review of the historical environmental monitoring data. Disinfectants applied on potential product contact surfaces are typically removed with 70% alcohol wipes. The removal of residual disinfectants should be monitored for effectiveness as a precaution against the possibility of product contamination.

The greatest safety concerns are in the handling of concentrated disinfectants and the mixing of incompatible disinfectants. For example, concentrated sodium hypochlorite solutions (at a concentration of more than 5%) are strong oxidants and will decompose on heating, on contact with acids, and under the influence of light, producing toxic and corrosive gases including chlorine. In contrast, dilute solutions (at a concentration of less than 0.5%) are not considered as hazardous. Under no circumstances should disinfectants of different concentrations be mixed. Material Safety Data Sheets for all the disinfectants used in a manufacturing area should be available to personnel handling these agents. Appropriate safety equipment such as face shields, safety glasses, gloves, and uniforms must be issued to personnel handling the disinfectant preparation, and personnel must be trained in the proper use of this equipment. Safety showers and eye wash stations must be situated in the work area where disinfectant solutions are prepared.

⟨1074⟩ EXCIPIENT BIOLOGICAL SAFETY EVALUATION GUIDELINES

INTRODUCTION

This informational chapter presents a scientifically-based approach for the safety assessment of new pharmaceutical excipients (i.e., those excipients that have not been previously used or permitted for use in a pharmaceutical preparation). The guidelines presented herein provide a protocol for developing an adequate database upon which to establish conditions for the safe use of a new excipient intended for use in products administered by various dosage routes. [NOTE—The final section of this chapter, *Definition of Terms*, lists some terms referred to in this chapter.]

An excipient may perform a variety of functionality roles in a pharmaceutical product; but, unlike pharmacologically active drug entities, the excipient displays either no pharmacological activity or very limited and directed activity. Because of these differences between excipients and active drug substances in terms of risk and benefit relationships and expected biological activities, the approaches for safety assessments of excipients and active drug substances will differ. Therefore, it is important to note that the guidelines presented in this informational chapter apply only to the safety assessment of excipients, not to the safety assessment of active drug substances.

These testing guidelines are informational in nature and are intended to be used by professionals having a knowledge of toxicology and associated sciences. It is also intended that the applicable safety test method requirements of the receiving regulatory authority would be used in a proposal for market entry. For example, if a proposal is to be submitted to the U.S. Food and Drug Administration, that agency's safety test requirements would have to be met. These guidelines do not provide specific details regarding test methodology and data interpretation. Test procedures that are generally recognized by experts and by the regulatory agencies should be used. Alternatives to the use of living animals are encouraged wherever these alternative procedures have been validated for the intended purpose and where it is known that the alternative procedure will provide sufficient data upon which to base a safety judgment. It is recommended that the *Guiding Principles on the Use of Animals in Toxicology* of the Society of Toxicology (1996) and, in other countries, the appropriate legal and professional codes, be adhered to in the conduct of all test procedures. All studies must meet the requirements of the appropriate national good laboratory practice guidelines in effect in the country where the studies are being conducted.

In cases of extensive human experience based upon food use, there may be sufficient information to fulfill the requirements of the guidelines for orally-ingested excipients only. In addition, there may be animal-based data, which was developed for other purposes, that may be used to fulfill the testing guidelines requirements. If the data requirements have been met through prior human use experience and pertinent human data have been collected in a scientifically sound manner, there is no need to provide animal data for those endpoints evaluated by prior clinical experience.

Some dosage routes offer unique toxicological challenges, and the guidelines include provisions for these routes (e.g., inhalation). Also, further explanation is provided regarding numbers of species and other basic information (e.g., two species, one rodent and one nonrodent).

The extent of information required to define a set of baseline data, which constitute a toxicological and chemical database, is dependent upon the intended use of, and duration of, dosing of the candidate excipient material. It is critical that a thorough review of background information be conducted before embarking on a testing regimen. In addition to literature database reviews, information should be obtained regarding the physical and chemical properties of the compound; its manufacturing process (or processes); and product specifications including limits of impurities, potential for pharmacological activity, exposure conditions (i.e., dose, duration, frequency of use, dosage formulation, and route of administration), and potential user population. Also, base toxicity information covering the topics is fundamental. Particular attention should be addressed to the absorption/distribution/metabolism/excretion/pharmacokinetics (ADME/PK) studies because much of the later decision process will be dependent upon these data.

These guidelines provide a mechanism for obtaining sets of baseline data for all candidate excipient materials. The background information and baseline toxicity information alone may support the use of the candidate excipient either in a short half-life product that is not administered in a frequency that results in a residual excipient build-up in body tissue or in a product used only once or twice in a lifetime, such as a diagnostic agent. Additional tests, listed under *Step 4* of the *Safety Assessment Guidelines*, are necessary for candidate excipient material that is to be used in a manner that will result in short- or intermediate-term repeated exposure in humans—that is, a pharmaceutical product that will be administered for less than 10 days or for 30 to 90 consecutive days, respectively. For a candidate excipient material that is intended for use in a pharmaceutical product intended for either intermittent or chronic administration over a long time period, such as a treatment for psoriasis or an insulin preparation, further tests are required. These tests are listed under *Step 7* of the guidelines and in the appropriate section under *Additional Requirements for Specific Exposure Routes*. While providing guidance for consumer safety, some of the required tests are intended to provide information to address occupational safety (e.g., skin and eye irritation).

The guidelines are summarized in *Table 1*. Tests that are required (R) by the guidelines are distinct from those that are recommended conditionally (C). Whether conditional tests are conducted is dependent upon the conditions of use and available biological data. Consideration must also be given to the requirements of the regulatory authorities when making the decision to test.

Table 1. Summary of Excipient Guidelines

	Routes of Exposure for Humans					
Tests	**Oral**	**Mucosal**	**Dermal/ Topical/ Trans-dermal**	**Injectable***	**Inhalation/ Intranasal**	**Ocular**
Baseline Toxicity Data						
Acute Oral Toxicity	R	R	R	R	R	R
Acute Dermal Toxicity	R	R	R	R	R	R
Acute Inhalation Toxicity	C	C	C	C	R	C
Eye Irritation	R	R	R	R	R	R
Skin Irritation	R	R	R	R	R	R
Skin Sensitization	R	R	R	R	R	R
Acute Injectable Toxicity	—	—	—	R	—	—
Application Site Evaluation	—	—	R	R	—	—
Pulmonary Sensitization	—	—	—	—	C	—
Phototoxicity/Photoallergy	R	—	R	R	R	—
Genotoxicity Assays	R	R	R	R	R	R
ADME/PK-Intended Route	R	R	R	R	R	R
28-Day Toxicity (2 Species)-Intended Route	R	R	R	R	R	R
Additional Data: Short- or Intermediate-term Repeated Use						
90-Day Toxicity (Most Appropriate Species)	R	R	R	R	R	R

R = Required
C = Conditional
* Intravenous, intramuscular, subcutaneous, intrathecal, etc.

Table 1. Summary of Excipient Guidelines *(Continued)*

	Routes of Exposure for Humans					
Tests	**Oral**	**Mucosal**	**Dermal/ Topical/ Trans-dermal**	**Injectable***	**Inhalation/ Intranasal**	**Ocular**
Embryo-Fetal Toxicol.	R	R	R	R	R	R
Additional Assays	C	C	C	C	C	C
Genotoxicity Assays	R	R	R	R	R	R
Immunosupression Assays	R	C	C	R	C	C
Additional Data: Intermittent Long-term or Chronic Use						
Chronic Toxicity (Rodent, Nonrodent)	C	C	C	C	C	C
Reproductive Toxicity	R	R	R	R	R	R
Photocarcinogenicity	C	—	C	C	C	—
Carcinogenicity	C	C	C	C	C	C

R = Required
C = Conditional
* Intravenous, intramuscular, subcutaneous, intrathecal, etc.

SAFETY ASSESSMENT GUIDELINES

Background Information

Before proceeding to the steps under *Data Requirements and Checkpoints*, the following points should be reviewed and defined:

- Review literature information using all appropriate databases
- Define chemical and physical properties
- Define manufacturing process
- Define product specifications, including impurities and residual solvents (see applicable ICH guidelines)
- Estimate exposure conditions (dose, duration, frequency route)
- Define user population
- Assess potential for pharmacologic activity.

At this point evaluate what is known, and develop the initial approach to testing.

Data Requirements and Checkpoints

STEP 1

Toxicity Data (see *Baseline Toxicity Data*)
The toxicity data should take into account the following information:

- Effects of acute exposure by oral and intended routes
- Effects of repeated exposures by intended routes
- Effects of in vitro genotoxicity assays
- ADME/PK by oral or appropriate routes; single or multiple doses.

STEP 2

Depending on results of above, evaluate effects of a single dose in humans.

STEP 3

Checkpoint: Evaluate results of above and proposed exposure conditions and exposed population. The above data might allow use in a single product with a short half-life (e.g., a diagnostic agent).

STEP 4

Gather the following additional data:

- Effects of subchronic exposure in appropriate species and routes
- Embryo-fetal development studies via appropriate route of exposure
- Additional in vitro and in vivo genotoxicity tests.

STEP 5

Depending on results of above, consideration should be given to testing in humans as part of the clinical trials of an active ingredient or as a stand-alone procedure.

STEP 6

Checkpoint: Evaluate all of above information. Data might allow use in a variety of products intended for short-term, repeated intake (e.g., an antibiotic). If the ADME/PK studies for a noninjectable excipient show no absorption, data may permit using a product for 30 to 90 consecutive days.

STEP 7

Additional data should be obtained for use in a product taken chronically, either daily or intermittently, over a long time period depending on:
- Results of subchronic studies and long-term toxicity in appropriate mammalian nonrodents
- Reproductive toxicity studies
- Other test results and human exposure data and long-term toxicity or carcinogenicity in rodents.

Baseline Toxicity Data

The following data should be taken into account:

APPROPRIATE ACUTE TOXICITY BY INTENDED DOSE ROUTES

skin sensitization, approximate lethal dose method, limit test, etc.

OTHER APPROPRIATE ACUTE TOXICITY STUDIES

oral toxicity by limit test or approximate lethal dose method, skin irritation, etc.

ADME/PK

single or multiple doses.

GENOTOXICITY

for example, Ames Test, in vitro chromosome aberration test, mammalian cell mutation assay.

28-DAY REPEATED DOSING STUDIES IN TWO SPECIES BY APPROPRIATE ROUTES (ONE RODENT, ONE MAMMALIAN NONRODENT):

evaluation of injection site or similar considerations might be necessary depending on route of administration.

NOTES

1. In those cases where intended route restrictions (e.g., volume, concentration) preclude an adequate assessment of the toxicity of the excipient, development of a toxicity profile by other relevant routes may be needed.
2. The comparison of toxicity and ADME/PK data between oral and intended routes is critical at this point because that knowledge may set the direction for future toxicity testing (e.g., reproductive toxicity testing conducted by oral route rather than intended route). In addition, relevant studies using the intended route and anticipated duration of exposure may preclude performance of additional studies.

Additional Requirements for Specific Exposure Routes

FOR ORAL EXPOSURE

No additional requirements beyond those presented for *Baseline Toxicity Data.*

FOR MUCOSAL EXPOSURE

No additional requirements beyond those presented for *Baseline Toxicity Data.*

FOR DERMAL, TOPICAL, OR TRANSDERMAL EXPOSURE

Baseline Toxicity Data

- *Effects of Acute Exposure by Transdermal Dose Route:* dermal sensitization study for repeat applications
- *Effects of Repeated Exposures by Transdermal Route*
  1. Photoallergy/phototoxicity study
  2. Studies in two species (one rodent, one mammalian nonrodent) by transdermal route.
- *Effects of Subchronic Exposure, Reproductive Toxicity Effects*—Initial toxicity studies may be performed by the IV route to adequately profile the toxicity of the excipient. This will provide an assessment of potential target organs if an adequate amount of the compound cannot be delivered via a transdermal dosage form. This is dependent upon the results from the ADME/PK studies.

Reproductive studies may also be conducted via oral or IV route with demonstration of absorption (oral) and pharmacokinetic comparisons of the chosen route versus transdermal.

Photocarcinogenicity studies may be required and should be considered if data and the proposed use indicate when evaluating materials to be placed on the skin for prolonged periods of time and exposure to UV light is a factor (e.g., sun block). This also applies to oral, parenteral, and inhalation products where skin drug concentrations exceed plasma drug concentrations for a substantial period of time, or where the candidate material would appear to have the potential for photo-activity or has demonstrated photo-activity.

FOR INJECTABLE DOSAGE FORMS

Background Information

1. Define compatibility of the dosage form with blood, if appropriate, based on route of exposure.
2. Define the pH and tonicity of injectable dose form, if appropriate, based on the route of exposure.

Baseline Toxicity Data

- *Effects of Acute Exposure by Intended Injectable Dose Routes*
  1. Include evaluation of injection site irritation in rabbit or dog
  2. Include evaluation of rate of administration.

FOR INHALATION OR INTRANASAL EXPOSURE[1]

Baseline Toxicity Data

- *Acute Inhalation Toxicity*—A limit test that would, for example, use the highest achievable concentration in a 4-hour exposure to vapor, aerosol, or solid particulate. Pulmonary sensitization may be performed along with other appropriate studies. If exposure is to be to an aerosol or solid particulate, particulates of appropriate mass median diameter should be generated.
- *Single and Repeated Dose ADME/PK by Inhalation or Intranasal and Oral Routes*
- *28-Day Repeated Dose Inhalation Study in Two Mammalian Species Using Vapor or Particulates of Appropriate Mass Median Diameter:* compare to similar oral toxicity data.

FOR OPHTHALMIC EXPOSURE

Background Information: define pH and osmolarity of topical ocular dose form.

Baseline Toxicity Data

- *Effects of Acute Exposure by Ophthalmic Routes:* cytotoxicity tests (e.g., agar overlay)

[1] When designing studies to evaluate use in products intended for use by the inhalation or intranasal route, consideration should be given to the dosing regimen that will be used by humans. The appropriate study protocol for a product intended for inhalation therapy that will result in prolonged exposures (e.g., several hours per day) may differ from that used to evaluate a product that would result in exposure to several metered doses per day.

- *Effects of Repeated Exposures by Ophthalmic Routes*
  1. Studies in two species (one rodent, one mammalian nonrodent)
  2. Examination of anterior and posterior segments of the eye
  3. Studies on allergenicity potential.

Other Data—Comparison of pharmacokinetic parameters of the route chosen for reproductive studies and the ophthalmic exposure are essential for extrapolation of potential toxicity via the ophthalmic route.

GLOSSARY

Acute: exposure to a test agent within a single, 24-hour period. Doses may be single, multiple or continuous during a 24-hour period.
Subacute: repeated dosing of a test agent for up to 29 days. Daily doses may be single, multiple or continuous during a 24-hour period.
Subchronic: repeated dosing of a test agent for 30 days to 10% of the lifespan of the test species (90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.
Chronic: repeated dosing of a test agent for more than 10% of the lifespan of the test species (more than 90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

⟨1078⟩ GOOD MANUFACTURING PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

BACKGROUND

This general information chapter provides guidelines for methods, facilities, and manufacturing controls to be used in the production of pharmaceutical excipients in order to ensure that excipients possess the quality, purity, safety, and suitability for use that they purport to possess. The principles and information in this chapter can be applied to the manufacture of all pharmaceutical excipients (referred to throughout this document as *excipient[s]*) intended for use in human drugs, veterinary drugs, and biologics. It covers the quality management system and the extent of good manufacturing practices (GMP) necessary throughout manufacturing for both batch and continuous processes. It is intended to assist manufacturers as well as auditors in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality and purity that they purport to possess and are suitable for their intended use. The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients for parenteral, ocular, inhalation, and open wound use and those that are sterile and/or pyrogen-free. It does not provide information for all national legal requirements nor does it cover in detail the particular characteristics of every excipient. The quality system standard used as a framework for this chapter is ISO 9001, which is appropriate to manufacturing. Because of the diversity of excipients, some principles in this information chapter may not be applicable to certain products and processes.

This chapter combines the concepts of existing GMP principles from the following sources:

- World Health Organization (WHO) GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC) Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients,
- International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, deference to all schemes is becoming necessary. Therefore, relevant portions of the manufacturing concepts are employed throughout this chapter.

The *General Guidance* section provides an overview of the appropriate manufacturing practice criteria applicable to excipient manufacture and the points of application of excipient good manufacturing practices and quality systems. The section also recommends measures to limit contamination of an excipient. Finally, it discusses the relationship of excipients to finished dosage forms. No attempt has been made to include details specific to particular excipients.

The information in *Appendix: Auditing Considerations* sets forth key criteria to aid in the audit of an excipient manufacturing facility.

For a list of terms used in this chapter and their definitions, see *Glossary*.

INTRODUCTION

Purpose and Scope

This chapter defines the extent and point of application of appropriate GMP principles for excipient manufacture and is applicable to the manufacture of excipients intended for use in drug products. It covers the quality management system and the extent of GMP necessary throughout manufacturing for both batch and continuous processes. It is intended to aid both auditors and manufacturers in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality, purity, and safety that they purport to possess and are suitable for their intended use.

The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients

- for parenteral, ocular, inhalation, and open wound use; and
- those that are purported to be sterile and/or pyrogen-free.

In these cases, detailed information pertaining to the intended use of an excipient as provided by the end user can be useful in determining appropriate GMP. This chapter does not address the specific GMP relating to good trade and distribution practices (GTDP).

Principles Adopted

THE CHAPTER AND ITS USE

Pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. Each manufacturer should consider how the chapter might apply to its products and processes (for example, batch versus continuous processes). Because excipients are so diverse, some principles of this chapter may not be applicable to certain products and manufacturing processes.

APPLICATION

The text provides information necessary for the manufacture of excipients but does not provide all the details. It cannot specify national legal requirements or cover particular characteristics of every excipient.

QUALITY SYSTEM STANDARD

The quality management system standard chosen as a framework for this chapter is ISO 9001, which is appropriate for manufacturing facilities. A manufacturer may apply the ISO standard with or without certification; but this possibility, as a business decision, is not discussed in this chapter. However, ISO certification has the benefit of providing assurance to customers that the excipient manufacturer's quality management system has been independently verified.

The headings in this chapter have been aligned with the ISO 9001 clause numbers, because many excipient manufacturers already use that standard as a basis for their quality management system. Additional headings are included as needed to introduce additional guidance on GMP when not covered by current ISO 9001 clauses.

DOCUMENT STRUCTURE

The chapter combines the concepts of existing GMP principles from the following:

- World Health Organization (WHO), GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC), Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients,International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, relevant portions of the manufacturing concepts detailed in these schemes are employed throughout this chapter.

The *General Guidance* section provides an overview of the GMP criteria applicable to excipient manufacture and the point of application of excipient GMP.

The remaining sections provide guidance on GMP principles and implementation of a quality management system suitable for excipient manufacture. For example, these sections suggest measures to limit excipient contamination. No attempt has been made to include details specific to particular excipients, and individual manufacturers should address these as they apply to their own products and processes.

The Appendixes provide supporting guidance for excipient GMP. *Appendix: Auditing Considerations* describes key criteria to be considered in the audit of an excipient manufacturing facility. *Glossary* provides definitions of terms used in this chapter.

GENERAL GUIDANCE

Pharmaceutical Excipients

Pharmaceutical excipients are substances other than the active pharmaceutical ingredient (API) that have been appropriately evaluated for safety and are intentionally included in a drug delivery system. For example, excipients can do the following:

- aid in the processing of the drug delivery system during its manufacture,
- protect, support, or enhance stability, bioavailability, or patient acceptability,
- assist in product identification, and
- enhance any other attribute of the overall safety, effectiveness, or delivery of the drug during storage or use.

A more complete classification of excipients according to their functions can be found in *USP and NF Excipients, Listed by Category* in the *USP–NF*.

Excipient GMP Implementation

The application of GMP is relevant once it has been determined that a chemical is intended for use as a component of a drug product. Excipient manufacture should be carried out in accordance with the GMP concepts consistent with this chapter. The objective of excipient GMP is to ensure that the manufacture of an excipient results in a consistent material with the desired quality characteristics. The emphasis of GMP for excipients is to ensure product integrity, avoid product contamination, and ensure that records are maintained.

As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product should increase. Manufacturing processes should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in this chapter should be applied and maintained.

Judgment based on risk analysis and a thorough knowledge of the process is required in order to determine from which processing step GMP should be implemented. This is usually well before the final finishing operation and, for example, may be identified using methods such as hazard analysis and critical control point (HACCP), failure mode and effects analysis (FMEA), or a detailed process flow diagram. Consideration should also be given to other factors such as batch versus continuous processing, dedicated versus multipurpose equipment, and open versus closed processes.

QUALITY MANAGEMENT SYSTEM: EXCIPIENT QUALITY SYSTEMS

General Recommendations

The principles outlined in this chapter provide a comprehensive basis for the quality management system used in the manufacture of pharmaceutical excipients. Excipient manufacturers should identify the quality management processes required to ensure excipient quality. Where manufacturing, testing, or other operations that could affect excipient quality are outsourced, the responsibility for quality remains with the excipient manufacturer, and control measures should be defined (see also the subsection *Purchasing Information* in the *Product Realization* section).

Documentation Recommendations

GENERAL

The excipient manufacturer should have a system in place to control documents and data that relate to the requirements of the quality management system.

QUALITY MANUAL

The excipient manufacturer should prepare a quality manual describing the quality management system, the quality policy, and the commitment of the excipient manufacturer to applying the appropriate GMP and quality management standards contained in this chapter. This manual should include the scope of the quality management system, reference to supporting procedures, and a description of the interaction between quality management processes.

General Chapters

CONTROL OF DOCUMENTS

The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of controlled documents, including documents of external origin that are part of the quality management system.

Procedures used in the manufacture of excipients should be documented, implemented, and maintained. In addition, there should be formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is being used throughout the operational areas and that previous revisions of documents have been removed.

Documents and subsequent changes to documents should be reviewed and approved by designated qualified personnel before issuance to the appropriate areas, as identified in the documents. Documents that affect product quality should be reviewed and approved by the quality unit (see also *Responsibility and Authority* in the section *Responsibility, Authority, and Communication* under *Management Responsibility*).

Controlled documents may include a unique identifier, the date of issue, and a revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. When it is practical, changes and the reasons for the changes should be documented.

Electronic documentation should meet the requirements for the document control system stated above. If electronic signatures are used on documents, they should be controlled to provide security equivalent to that provided by a handwritten signature. Electronic documents and signatures may also have to satisfy local regulatory requirements.

CONTROL OF RECORDS

The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records.

Records should be maintained to demonstrate achievement of the required quality and the effective operation of the quality management system. Records should be legible and identifiable with the product involved. Pertinent subcontractor quality data should be an element of these records.

Entries in records should be clear, indelible, made directly after performing the activity (in the order performed), and signed and dated by the person making the entry. Corrections to entries should be signed and dated, leaving the original entry legible.

Records should be kept for a defined period. This period should be appropriate to the excipient and to its expiry date or reevaluation interval. Records should be stored and maintained in such a manner that they are readily retrievable, in facilities that provide an environment suitable for minimizing deterioration or damage.

CHANGE CONTROL

The excipient manufacturer should establish and maintain procedures to evaluate and approve changes that may affect the quality of the excipient. For example, this may include changes to the following:

- raw materials or packaging and their sources,
- material specifications,
- test methods,
- manufacturing and analytical equipment,
- production processes,
- manufacturing or packaging sites.

A unit with a function that is independent from production (such as regulatory affairs or quality assurance) should have the responsibility and authority for the final approval of changes.

Customers should be notified, and, where applicable, excipient regulatory submissions (for example, for Drug Master Files [DMFs] or Certificates of Suitability to the *European Pharmacopoeia* [CEPs]) should be amended to reflect significant changes from established production and process control procedures that may affect excipient quality (see also *Customer Communication* in the section *Customer-Related Processes* under *Product Realization*).

MANAGEMENT RESPONSIBILITY

Management Commitment

Top management should demonstrate to the organization the importance it places on customer satisfaction and compliance with the appropriate regulations and standards. This should be accomplished through the development of a quality policy and establishment of quality objectives. Progress toward the documented quality objectives should be reviewed at planned intervals.

Customer Focus

It is the responsibility of top management to ensure that customer requirements are determined and met. The excipient manufacturer should permit the customer or its representative to conduct audits of the manufacturer's quality management system, manufacturing processes, buildings, and facilities.

Quality Policy

Top management should demonstrate its commitment to the corporate quality policy and ensure that it is implemented within the operational unit. The quality policy should support continual improvement of the quality management system. Management should participate in the development of the company's quality policy and provide the resources necessary for its development, maintenance, and deployment.

Planning

QUALITY OBJECTIVES

Top management should set objectives for adherence to GMP to ensure that the excipient manufacturer maintains and improves its performance. Objectives should be deployed throughout the organization and should be measurable and consistent with the quality policy.

QUALITY MANAGEMENT SYSTEM PLANNING

Top management should provide adequate resources to ensure conformity to the provisions of this chapter. There should be a process for the identification of resources needed for adherence to GMP. A gap analysis based on audits by internal personnel, customers, regulatory agencies, or outside contractors, or based on the use of this chapter, could be created to identify resource requirements. Top management should ensure that the integrity of the quality management system is maintained when changes are planned and implemented.

Responsibility, Authority, and Communication

RESPONSIBILITY AND AUTHORITY

Responsibility and authority should be clearly defined by top management and communicated within the organization. It should be the responsibility of a unit that is independent of production, such as the quality unit, to do the following:

- ensure that quality-critical activities are undertaken as defined,
- approve suppliers of quality-critical materials and services,
- approve or reject raw materials, packaging components, intermediates, and finished excipients,
- ensure that there is a review of production records to confirm that no errors have occurred or, if errors have occurred, that they are fully investigated,
- participate in reviewing and authorizing changes to processes, specifications, procedures, and test methods that potentially affect quality (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) and participate also in investigating failures and complaints,
- retain responsibility for approval or rejection of the excipient if it is produced, processed, packaged, or held under contract by another company,
- develop and implement a self-inspection program of the quality management system.

The excipient manufacturer may delegate some of the quality unit's activities to other personnel if appropriate controls (for example, periodic audits, training, and documentation) are in place.

An organization chart by function should show interdepartmental relationships as well as relationships to top management of the company. Personnel whose positions affect excipient quality should have job descriptions.

MANAGEMENT REPRESENTATIVE

The excipient manufacturer should appoint a management representative with sufficient authority to ensure that the provisions of this chapter are properly implemented. The representative should periodically report to top management on conformity to the quality management system, including changing customer and regulatory requirements.

INTERNAL COMMUNICATION

The excipient manufacturer should ensure that appropriate systems are established to communicate GMP and regulatory requirements, quality policies, quality objectives, and procedures throughout the organization. The communication should also provide information about the effectiveness of the quality management system. Top management should be notified promptly of quality-critical situations, such as product retrievals, in accordance with a documented procedure.

Management Review

GENERAL

The top management of the company should hold periodic reviews of the quality management system to confirm the organization's continued conformity to this chapter. The review should be recorded and should include assessing opportunities for improvement and the need for changes to the quality management system.

REVIEW INPUT

Management review inputs should include, for example, the following:
- results of internal and external audits,
- customer feedback of the company performance,
- product conformity and process performance,
- action items from the previous management review,
- customer complaints,
- status of corrective or preventive actions,
- changes that could affect the quality management system.

REVIEW OUTPUT

The management review should identify the resources needed and the opportunities presented for improving the quality management system and improving product conformity to customer and regulatory requirements. A record should be made of actions recommended and taken.

RESOURCE MANAGEMENT

Provision of Resources

There should be sufficient qualified personnel and resources (e.g., equipment, materials, buildings, and facilities) to implement, maintain, and improve the quality management system and to produce, package, test, store, and release each excipient in a manner consistent with this chapter.

Human Resources

GENERAL

Personnel performing work affecting the quality of excipients should have the appropriate combination of education, training, and experience for their assigned tasks. Consultants advising on the design, production, packaging, testing, or storage of excipients should have sufficient education, training, and experience or any combination thereof to advise on the subject for which they are retained. Records should be maintained listing the name, address, and qualifications of consultants and the type of service they provide.

COMPETENCE, AWARENESS, AND TRAINING

The excipient manufacturer should establish and maintain procedures for identifying training needs and for providing the necessary training to personnel performing activities affecting excipient quality. Appropriate records of training should be maintained. Training should address the particular operations that the employee performs and GMP as they relate to the employee's functions. Qualified individuals should conduct GMP training frequently enough to ensure that employees remain familiar with applicable GMP principles. Management should establish adequate and continued personal-hygiene training for personnel who handle materials so that they understand the precautions necessary for preventing contamination of excipients. The training program should ensure that personnel understand that deviations from procedures may affect the customer's product quality.

PERSONNEL HYGIENE

To protect excipients from contamination, protective apparel such as head, face, hand, and arm coverings should be worn as appropriate to the duties performed. Jewelry and other loose items, including those in pockets, should be removed or covered. Only authorized personnel should enter the areas of the buildings and facilities designated as limited-access areas.

Personnel should practice good sanitation and health habits. Any person shown by either medical examination or supervisory observation to have an apparent illness or open lesions that may adversely affect the safety or quality of the excipient should be excluded from direct contact with raw materials, packaging components, intermediates, and finished excipients until the condition is corrected or until competent personnel determine that it will not jeopardize the safety or quality of the excipient. Personnel should be instructed to report to supervisory personnel any health conditions that may have an adverse effect on excipients. The storage and use of food, drink, personal medication, tobacco products, or similar items should be restricted to designated locations separate from manufacturing areas.

INFRASTRUCTURE

The infrastructure should be managed, operated, cleaned, and maintained in accordance with GMP principles to ensure excipient quality and to avoid contamination (including, where critical to excipient quality, control of particulate matter, microbiological control, and control of water quality).

BUILDINGS AND FACILITIES

The prevention of contamination should be considered in the design of the manufacturing processes and facilities, particularly when the excipient is exposed. Buildings and facilities used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation appropriate to the type of processing.

Manufacturing processes associated with the production of highly sensitizing or toxic products (for example, herbicides and pesticides) should be located in dedicated facilities or should use equipment separate from that used for excipient manufacture. If this is not possible, appropriate measures (for example, cleaning, inactivation) should be implemented to avoid cross-contamination. The effectiveness of these measures should be demonstrated. There should be adequate facilities for the testing of raw materials, packaging components, intermediates, and finished excipients.

EQUIPMENT

Equipment used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation, depending on the type of processing (for example, batch versus continuous). Equipment should be commissioned before use to ensure that it is functioning as intended. Where equipment is located outdoors, there should be suitable controls to minimize the risk to excipient quality from the environment (for example, processing within a closed system).

EQUIPMENT CONSTRUCTION

Process equipment should be constructed so that contact surfaces will not be reactive, additive, or absorptive and thus will not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, should preferably not come into contact with raw materials, packaging materials, intermediates, or finished excipients. Where contact is possible, substances suitable for use in food applications should be employed.

Equipment should be designed to minimize the possibility of contamination caused by direct operator contact in activities such as the unloading of centrifuge bags, the use of transfer hoses (particularly those used to transfer powders), and the operation of drying equipment and pumps. The sanitary design of transfer and processing equipment should be evaluated. To control the risk of contamination, equipment with moving parts should be assessed with regard to the integrity of seals and packing materials.

EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the production, processing, packaging, testing, or holding of the excipient. There should be records of the use and maintenance of quality-critical equipment. These records can be in the form of a log, computer database, or other appropriate documentation.

COMPUTER SYSTEMS

Computer systems that may affect excipient quality should have sufficient controls for operation and maintenance and for prevention of unauthorized access or changes to computer software, hardware, or data, including the following:

- systems and procedures that show that the equipment and software are performing as intended,
- procedures for checking the equipment at appropriate intervals,
- retention of suitable back-up or archival systems such as copies of the program and files,
- assurance that changes are verified and documented and are made only by authorized personnel.

UTILITIES

Utilities (for example, nitrogen, compressed air, and steam) used in the production, storage, or transfer of materials that could affect excipient quality should be assessed and appropriate action taken to control the risk of contamination and cross-contamination.

WATER

Water used in the manufacture of excipients should be demonstrated to be of appropriate quality in consideration of purity requirements and the intended use of the excipient. Unless otherwise justified, process water should, at a minimum, meet regulatory requirements for drinking (potable) water. If drinking (potable) water is insufficient to ensure quality, or if tighter chemical and/or microbiological water quality specifications are required, appropriate controls and specifications should be set: for example, physical and chemical attributes, total microbial counts, and limits on objectionable organisms and/or endotoxins.

Where water used in the process is treated by the manufacturer to achieve a defined quality, the treatment process should be specified and monitored with appropriate action limits. Water that comes into contact with the excipient should be supplied under continuous positive pressure (or other means of preventing back flow) in a system free of defects to control the risk of contamination to the excipient.

WORK ENVIRONMENT

Where the excipient is exposed during manufacture, it should be in an environment appropriate for minimizing contamination. The manufacturer should apply suitable controls to maintain that environment.

AIR HANDLING

Where an air-handling system is installed to provide protection to the excipient, the excipient manufacturer should demonstrate its effectiveness. Excipient production unit air-handling systems should be designed to prevent cross-contamination. For dedicated areas processing the same excipient, it is permissible to recycle a portion of the exhaust air back into the same area. The adequacy of such a system for multiuse areas, especially if several products are processed simultaneously, should be assessed for potential cross-contamination.

CONTROLLED ENVIRONMENT

A controlled environment may be necessary in order to avoid contamination or degradation caused by exposure to heat, air, or light. The degree of protection required may vary depending on the stage of the process. Special environments required by some processes should be monitored to ensure product quality (for example, inert atmosphere or protection from light). Where an inert atmosphere is required, the gas should be treated as a raw material. If interruptions in the special environment occur, adequate evidence and an appropriate rationale should be documented to show that such interruptions have not compromised the quality of the excipient. Such environmental concerns become increasingly important following purification of the excipient.

CLEANING AND SANITARY CONDITIONS

Adequate cleanliness is an important consideration in the design of excipient manufacturing facilities. Buildings used in the production, processing, packaging, or holding of an excipient should be maintained in an appropriately clean and sanitary condition according to the type of processing conducted (for example, open/closed systems). Where maintenance of clean and sanitary conditions is critical to excipient quality, documented procedures should assign responsibility for cleaning and sanitation, describing in sufficient detail the cleaning schedules, methods, equipment, and materials to be used in cleaning the buildings and facilities. These procedures should be followed, and cleaning should be documented. Waste should be segrega-

ted and disposed of in a timely and appropriate manner. If waste is not disposed of immediately, it should be suitably identified.

PEST CONTROL

Buildings should be free from infestation by rodents, birds, insects, and other vermin. Some raw materials, particularly botanicals, may contain some unavoidable contamination, such as rodent or other animal filth or infestation. The manufacturer should have sufficient control methods to prevent the increase of such contamination or infestation in holding areas and its spread to other areas of the plant.

LIGHTING

Adequate lighting should be provided to facilitate cleaning, maintenance, and proper operations.

DRAINAGE

In areas where the excipient is open to the environment, drains should be of adequate size and, where connected directly to a sewer, should be provided with an air break or other mechanical device to prevent back-siphoning.

WASHING AND TOILET FACILITIES

Adequate personal washing facilities should be provided, including hot and cold water, soap or detergent, air dryers or single-service towels, and clean toilet facilities easily accessible to working areas. Adequate facilities for showering and/or changing clothes should be provided, where appropriate.

PRODUCT REALIZATION

Planning of Product Realization

The excipient manufacturer should plan and develop the processes and controls needed for product manufacture. These plans and controls should be appropriate to the production process, excipient specification, equipment, and facilities used in the manufacture of the product. Key aspects of the planning of a suitable process and its controls should include the following, as appropriate:

- documented testing programs, for quality-critical materials including excipients, that include appropriate specifications, sampling plans, and test and release procedures,
- generation and maintenance of records (also see above, *Control of Records* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) that provide evidence that these plans have been realized as intended and that enable traceability to be demonstrated (also see below in this section, *Traceability* under *Identification and Traceability*),
- provision of resources to implement these plans,
- environmental and hygiene control programs to minimize contamination.

Customer-Related Processes

DETERMINATION OF REQUIREMENTS RELATED TO THE PRODUCT

The excipient manufacturer should determine the excipient quality, labeling, and delivery requirements of the customer. Additional requirements, whether customer-specific, legal, or regulatory (for example, pharmacopeia material and general monographs), should be agreed on by both parties. Requirements not stated by the customer but necessary for specified or intended use, where known, should be considered.

REVIEW OF REQUIREMENTS RELATED TO THE PRODUCT

The excipient manufacturer and customer should mutually agree upon the requirements identified in the section above, *Determination of Requirements Related to the Product*, before supply commences. The manufacturer should have the facility and process capability to consistently meet the mutually agreed-upon specifications. Where the requirements determined in the section *Determination of Requirements Related to the Product* are changed, this review should be repeated before supply recommences.

CUSTOMER COMMUNICATION

There should be provision for providing accurate and pertinent communication to the customer. Master copies of documents such as specifications and technical reports should be controlled documents. Provision should be made for replying to customer inquiries, contracts, and order-handling requirements. Customer feedback and complaints should be documented. Customers should be notified of significant changes (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*).

DESIGN AND DEVELOPMENT

ISO 9001 includes requirements for ensuring control over design and development activities. It is recommended that companies involved in such activities follow the requirements of ISO 9001. Full GMP are not always applicable during the design and development of new excipients and/or manufacturing processes. However, development batches of excipients that are intended for use in drug products should be manufactured in accordance with the applicable provisions of this chapter.

Purchasing

PURCHASING PROCESS

Excipient manufacturers should have a system for selecting and approving suppliers of quality-critical materials and services (for example, subcontract manufacturers and laboratories). Supplier approval by the quality unit should require an evaluation of the supplier's quality management system, including adequate evidence that they can consistently meet agreed-upon specifications and maintain traceability. This may require periodic audits of the supplier's manufacturing facility. Records of these activities should be maintained. Materials should be purchased against an agreed specification from approved suppliers.

PURCHASING INFORMATION

Purchasing agreements should describe the material or service ordered, including, where critical to excipient quality, the following:

- the name, type, class, style, grade, item code number or other precise identification traceable to the raw material and packaging specifications,
- drawings, process requirements, inspection instructions and other relevant technical data, including requirements for approval or qualification of product, procedures, process equipment, and personnel,
- adherence to the appropriate sections of this chapter for relevant contract manufacturers or laboratories, and
- a statement to notify the excipient manufacturer of significant changes in quality-critical raw materials.

VERIFICATION OF PURCHASED PRODUCT

There should be procedures for the approval and release of quality-critical material. Upon receipt, quality-critical materials should be placed in quarantine and should not be used prior to acceptance. Effective quarantine can be established with suitable identifying labels, signs, and/or other manual documentation systems. When quarantine and stock control are managed with computer systems in lieu of a physical stock control, system controls should prevent the use of unreleased material. Quarantine may not be feasible for materials supplied via pipelines. In these cases the excipient manufacturer should establish an agreement with the supplier so that the manufacturer is notified of material that does not meet specification. Sampling activities should be conducted under defined conditions, in accordance with a defined sampling method and using procedures designed to prevent contamination and cross-contamination.

Quality-critical materials used in the manufacture of an excipient should be tested or otherwise verified prior to use. Verification should include availability and a check of the supplier certificate of analysis and, wherever feasible, at least an identification test. Testing schedules should be organized to separate routine tests from those that are performed infrequently or only for new suppliers. Bulk deliveries should have additional controls to ensure material purity and freedom from contamination (for example, dedicated tankers, tamper-evident seals, a certificate of cleaning, analytical testing, or audit of the supplier). These procedures, activities, and results should be documented.

Production and Service Provision

CONTROL OF PRODUCTION AND SERVICE PROVISION

Production activities should be carried out under controlled conditions (also see above, *Planning of Product Realization* under *Product Realization*). Specific examples of important controls, some of which may not be applicable to all excipient manufacturers, are illustrated in the following sections.

General Chapters

PRODUCTION INSTRUCTIONS AND RECORDS

Production instructions and records are required but may differ for the type of operation: for example, batch versus continuous processes. There should be a controlled document that describes how the excipient is produced (for example, master production instructions, master production and control records, or process definitions). For batch processes, an accurate reproduction of the appropriate master production instructions should be issued to the production area. For continuous processes, a current processing log should be available. Records should be available for each batch of excipient produced and should include complete information relating to the production and control of each batch. For continuous processes, the batch and its records should be defined (for example, based on time or defined quantity). Records may be in different locations but should be readily retrievable. Records for both batch and continuous processing, where critical to excipient quality, should include the following:

- date and time each step was completed or date and time log of key parameters,
- identification of persons performing and directly supervising or checking each significant step, operation or control parameter,
- identification of major equipment and lines used,
- material inputs to enable traceability: for example, batch number and quantities of raw material/intermediate and time it was added,
- in-process and laboratory control results,
- the quantity produced for the defined batch and a statement of the percentage of theoretical yield, unless not quantifiable (for example, as in some continuous processes),
- inspection of the packaging and labeling area before and after use,
- labeling control records,
- description of excipient product containers and closures,
- description of sampling performed,
- failures, deviations and their investigations,
- results of final product inspection.

EQUIPMENT CLEANING

The manufacturer should design and justify cleaning and sanitization procedures and provide evidence of their effectiveness. In multipurpose plants the use of the *model product* approach (groups of product of similar type) may be used in justifying a suitable procedure. Cleaning and sanitization procedures should be documented. They should contain sufficient detail to allow operators to clean each type of equipment in a reproducible and effective manner. There should be a record confirming that these procedures have been followed. Equipment and utensils should be cleaned and sanitized where critical to excipient quality and at appropriate intervals to prevent contamination and cross-contamination of the excipient. The cleaning status of equipment should be recorded appropriately.

Where multipurpose equipment is in use, it is important to be able to determine previous usage when investigating cross-contamination or the possibility of such contamination (also see below in this section, *Records of Equipment Use*). During a production campaign, incidental carryover frequently occurs, and it is usually acceptable because cleanup between successive batches of the same excipient is not normally required in order to maintain quality levels. Products that leave residues that cannot be effectively removed should be produced in dedicated equipment. For continuous processing, the frequency of equipment cleaning should be determined by the manufacturer and justified.

RECOVERY OF SOLVENTS, MOTHER LIQUORS, AND SECOND CROP CRYSTALLIZATIONS

Where solvents are recovered and reused in the same process or different processes, they should meet appropriate standards prior to reuse or mixing with other approved material. Mother liquors or filtrates containing recoverable amounts of excipient, reactants, or intermediates are frequently reused. Such processes should be documented in the production records or logs to enable traceability.

IN-PROCESS BLENDING OR MIXING

In-process blending or mixing to ensure batch uniformity or to facilitate processing should be controlled and documented. If the intent of the operation is to ensure batch uniformity, it should be performed so as to ensure homogeneous mixing of materials to the extent feasible and should be reproducible from batch to batch.

IN-PROCESS CONTROL

In-process inspection and testing, based on monitoring the process or actual sample analysis at defined locations and times, should be performed. Sampling methods should be documented to ensure that the sample is representative and clearly labeled. In-process samples should not be returned to production for incorporation into the final batch.

The results of in-process tests should be recorded and should conform to established process parameters or acceptable tolerances. Work instructions should define the procedure to follow and should indicate how to use the inspection and test data to control the process. There should be defined actions to be taken when the results are outside specified limits. Where approval to continue with the process is issued within the production department, the specified tests should be performed by trained personnel and the results recorded.

PACKAGING AND LABELING

Procedures should be employed to protect the quality and purity of the excipient when it is packaged and to ensure that the correct label is applied to all containers. Packaging and labeling operations should be designed to prevent mix-ups. Procedures should be implemented to ensure that the correct labels are printed and issued and that the labels contain the correct information. The procedure should also specify that excess labels are immediately destroyed or returned to controlled storage. Excess labels bearing batch numbers should be destroyed. Packaging and labeling facilities should be inspected immediately before use to ensure that materials that are not required for the next packaging operation have been removed. When excipients are labeled on the packaging line, packaged in preprinted bags, or bulk-shipped in tank cars, there should be documentation of the system used to satisfy the intent of the above procedures.

RECORDS OF EQUIPMENT USE

Records of quality-critical equipment use should be retained. These records should allow the sequence of cleaning, maintenance, and production activities to be determined.

VALIDATION OF PROCESSES FOR PRODUCTION AND SERVICE PROVISION

An important factor in the assurance of product quality includes the adequate design and control of the manufacturing process, because product testing alone is not sufficient to reveal variations that may have occurred. Each step of the manufacturing process should be controlled to the extent necessary for ensuring that the excipient meets established specifications. The concept of process validation is a key element in ensuring that these quality assurance goals are met. The process reactions, operating parameters, purification steps, impurities, and key tests needed for process control should be documented, thus providing the basis for validation.

The full validation program that is typically performed in the pharmaceutical industry may not always be carried out by the excipient manufacturer. However, the excipient manufacturer should demonstrate the consistent operation of each manufacturing process: for example, through process capability studies, development, and scale-up reports.

Identification and Traceability

TRACEABILITY

Quality-critical items (for example, raw materials, packaging materials, intermediates, and finished excipients) should be clearly identified and traceable through records. These records should allow traceability of the excipient both upstream and downstream. Identification of raw materials used in batch production processes should be traceable through the batch numbering system or other appropriate system. Identification of raw materials used in excipients produced by continuous processing should indicate the time frame during which a particular batch of raw material was processed through the plant. Excipient manufacturers should also have adequate knowledge about the origin of any raw materials derived from plant or animal matter.

Raw materials, including solvents, are sometimes stored in bulk tanks or other large containers, making precise separation of batches difficult. Nevertheless, the use of such materials and containers should be documented in production records.

INSPECTION AND TEST STATUS

There should be a system for identifying the inspection status of quality-critical items, including raw materials, packaging materials, intermediates, and finished excipients. Although storing materials in identified locations is preferred, any means that clearly identifies the test status is satisfactory. Continuously fed materials may need special consideration in order to satisfy these requirements.

LABELING

Labeling for excipient packages is subject to national and international regulatory requirements, which may include transportation and safety measures. At a minimum, labels should include the following:

- the name of the excipient and grade, if applicable,
- the excipient manufacturer's and/or distributor's name,
- the batch number from which the complete batch history can be determined,
- special storage conditions, if applicable.

CUSTOMER PROPERTY

The excipient manufacturer should establish and maintain procedures for verification, storage, and maintenance of customer-supplied materials intended for incorporation into the customer's excipient. Verification by the manufacturer does not relieve the customer of the responsibility of providing an acceptable material. Material that is lost or that is damaged or otherwise unsuitable for use should be recorded and reported to the customer. In this case, procedures should be in place for acceptable disposition and replacement of the material. The manufacturer should also make provisions for protecting other real and intellectual property that is provided by the customer (for example, test equipment, test methods, and specifications).

Preservation of Product

HANDLING, STORAGE, AND PRESERVATION

Excipients, intermediates, and raw materials should be handled and stored under appropriate conditions of temperature, humidity, and light so that their identity, quality, and purity are not affected. Outdoor storage of raw materials (for example, acids, other corrosive substances, explosive materials) or excipients is acceptable, provided that the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, and containers are adequately cleaned prior to opening and use. Records of storage conditions should be maintained if they are critical for the continuing conformity of the material to specifications.

PACKAGING SYSTEMS

An excipient packaging system should include the following features:

- documented specifications and examination or testing methods,
- cleaning procedures, where containers are reused,
- tamper-evident seals,
- containers that provide adequate protection against deterioration or contamination of the excipient during transportation and recommended storage,
- containers that do not interact with or contaminate the excipient,
- storage and handling procedures that protect containers and closures and minimize the risk of contamination, damage, or deterioration and that will avoid mix-ups (for example, between containers that have different specifications but are similar in appearance).

If returnable excipient containers are reused, previous labeling should be removed or defaced. If the containers are reused solely for the same excipient, previous batch numbers or the entire label should be removed or completely obliterated.

DELIVERY AND DISTRIBUTION

Identification and traceability of quality-critical aspects are required of excipient manufacturers. Distribution records of excipient shipments should be kept. These records should identify, by excipient batch, where and to whom the excipient was shipped, the amount shipped, and the date of shipment so as to facilitate retrieval if necessary. Where excipients are handled by a series of different distributors, it should be possible to trace them back to the original manufacturer, and not only to the previous supplier. The manufacturer should maintain the integrity and the quality of the product after final inspection and test. Where contractually specified, this protection should be extended to include delivery to the final destination. Excipients should be supplied only within their expiry and/or retest period.

CONTROL OF MEASURING AND MONITORING DEVICES

Measuring and test equipment, including computerized systems, identified as being quality-critical should be calibrated and maintained. This includes in-process instruments as well as test equipment used in the laboratory. The control program should include the standardization or calibration of instruments and equipment at suitable intervals in accordance with an established, documented program. This program should contain specific directions, schedules, limits for accuracy and precision, and provi-

sions for remedial action in the event that accuracy and/or precision limits are not met. Calibration standards should be traceable to recognized national or compendial standards as appropriate.

Instruments and equipment not meeting established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration status of quality-critical equipment should be known and verifiable to users.

MEASUREMENT, ANALYSIS, AND IMPROVEMENT

The organization should plan and implement the monitoring, measurement, and improvement activities that are required in order to demonstrate conformity of the excipient to customer requirements and to ensure conformity of the quality management system to this chapter. The organization should evaluate opportunities for improvements through the measurement and analysis of product and process trends.

Monitoring and Measurement

CUSTOMER SATISFACTION

The excipient manufacturer should establish measurement activities to assess customer satisfaction. Such measurements can include customer complaints, return of excipients, and customer feedback. This information should drive activities that strive to continuously improve customer satisfaction.

INTERNAL AUDIT

The excipient manufacturer should carry out a comprehensive system of planned and documented internal quality audits. These should determine whether quality activities comply with planned arrangements and should also determine the effectiveness of the quality management system. Audits should be scheduled on the basis of the status and importance of the activity. Audits and follow-up actions should be carried out in accordance with documented procedures. Audit results should be documented and discussed with management personnel having responsibility in the area audited. Management personnel responsible for the area audited should take corrective action on the nonconformities found. *Appendix: Auditing Considerations* will be of assistance in establishing an internal audit program.

MONITORING AND MEASUREMENT OF PROCESSES

The excipient manufacturer should identify the tests and measurements necessary for adequately controlling manufacturing and quality management system processes. When critical to excipient quality, techniques used to verify that the processes are under control should be established. When deviations from planned results occur, corrective action should be taken to ensure that the excipient meets requirements. Periodic reviews of key indicators such as process quality attributes and process failures should be conducted to assess the need for improvements.

MONITORING AND MEASUREMENT OF PRODUCT

The excipient manufacturer should establish the test methods and procedures to ensure that the product consistently meets specifications. Analytical methods should be suited to their purposes. The analytical methods may be those included in the current edition of the appropriate pharmacopeia or another accepted standard. However, the methods may also be noncompendial. If the excipient manufacturer claims that its product is in compliance with a pharmacopeia or an official compendium, then

- noncompendial analytical tests should be demonstrated to be equivalent to those in the compendia;
- the product should comply with applicable *USP* general chapters and notices.

LABORATORY CONTROLS

Laboratory controls should include complete data derived from tests necessary for ensuring conformity with specifications and standards, including the following:

- a description of the sample received for testing, together with the material name, a batch number or other distinctive code, and the date the sample was taken,
- a statement referencing each test method used,
- a record of raw data secured during each test, including graphs, chromatograms, charts, and spectra from laboratory instrumentation, identified to show the specific material and batch tested,
- a record of calculations performed in connection with the test,
- test results and how they compare with established specifications,

- a record of the person who performed each test and the date(s) the tests were performed.

There should be a documented procedure for the preparation of laboratory reagents and solutions. Purchased reagents and solutions should be labeled with the proper name, concentration, and expiry date. Records should be maintained for the preparation of solutions and should include the name of the solution, the date of preparation, and the quantities of material used. Volumetric solutions should be standardized according to an internal method or by using a recognized standard. Records of the standardization should be maintained.

Where used, primary reference reagents and standards should be appropriately stored and need not be tested upon receipt, provided that a certificate of analysis from the supplier is available. Secondary reference standards should be appropriately prepared, identified, tested, approved, and stored. There should be a documented procedure for the qualification of secondary reference standards against primary reference standards. The reevaluation period should be defined for secondary reference standards, and each batch should be periodically requalified in accordance with a documented protocol or procedure.

FINISHED EXCIPIENT TESTING AND RELEASE

Finished excipient testing should be performed on each batch to ensure that the excipient conforms to documented specifications. There should be a procedure to ensure that appropriate manufacturing documentation, in addition to the test results, is evaluated prior to release of the finished excipient. The quality unit should be responsible for the release of the finished excipient. For excipients produced by continuous processes, assurance that the excipient conforms to documented specifications may be achieved through the results of in-process testing or other process control records.

OUT-OF-SPECIFICATION TEST RESULTS

Out-of-specification (OOS) test results should be investigated and documented according to a documented procedure. Retest sample results may be used to replace the original test result only if it is demonstrated on the basis of a documented investigation that the original result is erroneous. When statistical analysis is used, both the original and retest data must be included. The OOS procedure should define which statistical techniques are to be used and under what circumstances. These same principles apply when the sample is suspected of not being representative of the material from which it was taken.

RETAINED SAMPLES

When practical, a representative sample of each batch of the excipient should be retained. The retention period should be appropriate to the expiry or reevaluation date. The retained samples should be stored and maintained in such a manner that they are readily retrievable in facilities that provide a suitable environment. The sample size should be at least twice the amount required to perform complete specification testing.

CERTIFICATES OF ANALYSIS

The organization should provide certificates of analysis to the required specification for each batch of excipient.

IMPURITIES

When possible, excipient manufacturers should identify and set appropriate limits for impurities. The limits should be based on appropriate safety data, limits as described in official compendia or other requirements, and sound GMP considerations. Manufacturing processes should be adequately controlled so that the impurities do not exceed such established limits. Many excipients are extracted from or purified using organic solvents. These solvents are normally removed by drying. It is important that excipient specifications include tests and limits for solvent residues.

STABILITY

Although many excipient products are stable and may not require extensive testing to ensure stability, the stability of excipients is an important factor in the overall quality of the drug product. For excipients that have been on the market for a long time, historical data may be used to indicate stability. Where historical data do not exist, a documented testing and/or evaluation program designed to assess the stability characteristics of the excipient should be undertaken. The results of such stability testing and/or evaluation should be used in determining appropriate storage conditions and retest or expiry dates. The testing program should include the following:

- the number of batches, sample sizes and test intervals,
- storage conditions for samples retained for testing,
- suitable stability-indicating test methods,
- storage of the excipient in containers that simulate the market container, where possible.

The stability of excipients may be affected by undetected changes in raw materials or subtle changes in manufacturing procedures or storage conditions. Excipients may also be shipped in a variety of packaging types that can affect their stability (for example, plastic or glass bottles, metal or plastic drums, bags, tank cars, or other bulk containers).

Some excipients may be available in different grades (for example, various molecular weights of a polymer or different monomer ratios, different particle sizes, bulk densities) or may be mixtures of other excipients. These excipients may be very similar to others within a product group. Minor quantitative differences of some of the components may be the only significant variation from one product to another. For these types of excipients, a model product approach may be appropriate for assessment of the stability of similar excipients. Stability studies of this type should involve selection of several model products that would be expected to simulate the stability of the product group being assessed. This selection should be scientifically sound and documented. Data from stability studies of these model products can be used to determine theoretical stability for similar products.

EXPIRY/RETEST PERIODS

An expiry or retest period should be assigned to each excipient and communicated to the customer. Common practice is to use a retest period rather than an expiry period.

CONTROL OF NONCONFORMING PRODUCT

Raw material, intermediate, or finished excipient found not to meet its specifications should be clearly identified and controlled to prevent inadvertent use or release for sale. A record of nonconforming product should be maintained. Incidences of nonconformity should be investigated to identify the cause. The investigation should be documented and action taken to prevent recurrence. There should be a documented procedure defining how the retrieval of an excipient from distribution should be conducted and recorded. Procedures should exist for the evaluation and subsequent disposition of nonconforming products. Nonconforming product should be reviewed in accordance with documented procedures to determine if it can be

- reprocessed or reworked to meet the specified requirements,
- accepted by the customer with customer agreement,
- regraded for other applications,
- destroyed.

REPROCESSING

Repetition of an activity that is a normal part of the manufacturing process (reprocessing) should occur only when it has already been documented that the excipient may be made in that manner. In all other cases, the guidance for reworking should be followed.

REWORKING

An activity that is not a normal part of the manufacturing process (reworking) should be conducted only following a documented review of risk to excipient quality and approval by the quality unit. As appropriate, when performing the risk assessment, consideration should be given to the following:

- new impurities that may be introduced as a result of reworking,
- additional testing to control the reworking,
- records and traceability to the original batches,
- suitable acceptance criteria for the reworked excipient,
- impact on stability or the validity of the reevaluation interval,
- performance of the excipient.

When the need to rework an excipient is identified, an investigation and evaluation of the cause are required. The equivalence of the quality of reworked material to original material should also be evaluated and documented to ensure that the batch will conform to established specifications and characteristics. Batches of excipients that do not conform to specifications individually must not be blended with other batches that do conform in an attempt to hide adulterated or substandard material.

RETURNED EXCIPIENTS

Returned excipients should be identified and quarantined until the quality unit has completed an evaluation of their quality. There should be procedures for holding, testing, reprocessing, and reworking of the returned excipient. Records for returned products should be maintained and should include the name and the batch number of the excipient, the reason for the return, the quantity returned, and the ultimate disposition of the returned excipient.

ANALYSIS OF DATA

The excipient manufacturer should develop methods for evaluating the effectiveness of its quality management system and use those data to identify opportunities for improvement. Such data can be derived from customer complaints, product reviews, process capability studies, internal audits, and customer audits. The analysis of such data may be used as part of the management review (also see above, *Management Review* under the *Management Responsibility* section). A periodic review of key indicators such as product quality attributes, customer complaints, and product nonconformities may be conducted to assess the need for improvements.

Improvement

CONTINUAL IMPROVEMENT

The excipient manufacturer should take proactive measures to continuously improve manufacturing and quality management system processes. To identify opportunities for continual improvement, analysis of the following performance indicators may be considered:

- causes of nonconforming product,
- results of internal and external audits,
- customer returns and complaints,
- process and operational failures.

Corrective Action: The excipient manufacturer should establish, document, and maintain procedures for the following:

- determining the root causes of nonconformities,
- ensuring that corrective actions are implemented and effective,
- implementing and recording changes in procedures resulting from corrective action.

Preventive Action: The excipient manufacturer should establish, document, and maintain procedures for the following:

- initiating preventive actions to deal with problems at a level corresponding to the risks,
- implementing and recording changes in procedures resulting from preventive action.

GLOSSARY

The terms below are defined as used in this chapter. Wherever possible, definitions used by the International Conference on Harmonization have been used as the basis for the glossary.

Acceptance Criteria: numerical limits, ranges, or other suitable measures of acceptance for test results.

Active Pharmaceutical Ingredient (API): any substance or mixture of substances that is intended to be used in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

Adulterated Material: a material that either has been contaminated with a foreign material or has not been manufactured using GMP. This does not pertain to a material that simply does not meet physical or chemical specifications.

Batch (Lot): a specific quantity of material produced in a process or series of processes so that it can be expected to be homogeneous. In the case of continuous processes, a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval.

Batch Number (Lot Number): a unique combination of numbers, letters, and/or symbols that identifies a batch and from which the production and distribution history can be determined.

Batch Process: a process that produces the excipient from a discrete supply of raw materials that are present before the completion of the reaction.

Batch Record: documentation that provides a history of the manufacture of a batch of excipient.

Blending (Mixing): intermingling different conforming grades into a homogeneous lot.

Calibration: the demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard, over an appropriate range of measurements.

CEP (Certificate of Suitability to the *European Pharmacopoeia*): certification granted to individual manufacturers by the European Directorate for the Quality of Medicines (EDQM) when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

Certificate of Analysis: a document listing the test methods, specification, and results of testing a representative sample from the batch to be delivered.

Commissioning: the introduction of equipment for use in a controlled manner.

Contamination: the undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

Continuous Process: a process that continuously produces material from a continuing supply of raw material.

Critical: a process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.
Cross-Contamination: contamination of a material or product with another material or product.
Customer: the organization receiving the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.
Deviation: departure from an approved instruction or established standard.
Drug Master File (DMF): detailed information about the manufacture of an excipient that is submitted to the U.S. Food and Drug Administration (FDA).
Drug (Medicinal) Product: the dosage form in the final immediate packaging intended for marketing.
Excipient: substances other than the API that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.
Expiry (Expiration) Date: the date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.
Impurity: a component of an excipient that is not intended to be present but arises as a consequence of the manufacturing process.
In-Process Control/Testing: checks performed during production to monitor and, if appropriate, to adjust the process and/or to ensure that the intermediate or excipient conforms to its specification.
Intermediate: material that must undergo further manufacturing steps before it becomes an excipient.
Lot: See *Batch*.
Manufacturer/Manufacturing Process: all operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.
Master Production Instruction (Master Production and Control Record): documentation that describes the manufacture of the excipient from raw material to completion.
Material: a general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.
Model Product: a product that represents a group of similar products with respect to composition, functionality, or specification.
Mother Liquor: the residual liquid that remains after crystallization or isolation processes.
Packaging Material: a material intended to protect an intermediate or excipient during storage and transport.
Production: operations involved in the preparation of an excipient from receipt of materials through processing and packaging of the excipient.
Quality Assurance: the sum total of the organized arrangements made with the object of ensuring that all excipients are of the quality required for their intended use and that quality systems are maintained.
Quality Control: checking or testing that specifications are met.
Quality-Critical: describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.
Quarantine: the status of materials isolated physically or by other effective means pending a decision on their subsequent approval or rejection.
Raw Material: a general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.
Record: a document stating results achieved and/or providing evidence of activities performed. The medium may be paper, magnetic, electronic or optical, photographic, or another medium, or a combination thereof.
Reevaluation Date (Retest Date): the date when the material should be reexamined to ensure that it is still in conformity with the specification.
Reprocessing: repetition of an activity that is a normal part of the manufacturing process and that has been documented previously.
Retrieval: process for the removal of an excipient from the distribution chain.
Reworking: subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.
Specifications: list of tests, references to analytical procedures, and appropriate acceptance criteria that are numerical limits, ranges, or other criteria for the tests described for a material.
Stability: continued conformity of the excipient to its specifications.
Top Management: person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and will depend on the way in which the quality management system is organized.
Traceability: ability to determine the history, application, or location that is under consideration: for example, origin of materials and parts, processing history, or distribution of the product after delivery.
Validation: a documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

General Chapters

APPENDIX
Auditing Considerations

INTRODUCTION

Many excipients are used in food, cosmetic, and industrial products as well as in pharmaceuticals. Thus, environmental conditions, equipment, and operational techniques employed in excipient manufacture are often those of the chemical industry as opposed to the pharmaceutical industry. Chemical processes can produce impurities from side reactions. Careful process control is therefore essential to minimize levels of impurities and contamination.

Excipients are often manufactured on a large scale, using continuous processing and automated process controls. Production equipment and processes vary depending on the type of excipient being produced, the scale of production, and the type of operation (for example, batch versus continuous process).

This appendix is intended as an aid in preparing for an audit of an excipient manufacturer. Both external and internal auditors (see also *Internal Audit* in *Monitoring and Measurement* under the *Measurement, Analysis, and Improvement* section) will find this appendix useful in identifying the significant issues with respect to GMP and quality that require examination. This section will assist excipient manufacturers in identifying key deliverables when adopting the GMP standards listed in the other sections of this chapter; in planning an audit, it will also help to verify the quality of the excipient manufacturing process and the manufacturer's quality management system.

GMP PRINCIPLES

Control of Impurities and Contamination: In general, the pharmaceutical customer does not perform further chemistry or purification steps on the excipient; it is used as purchased. Consequently, impurities present in the excipient are likely to be present in the drug product. Although dosage form manufacturers have some control over excipient quality through specifications, excipient manufacturers have greater control over the physical characteristics, quality, and presence of impurities in the excipients they produce.

External contamination of the excipient can arise from the manufacturing environment. However, chemical processes used to manufacture excipients are often performed in closed systems that afford protection against such contamination, even when the reaction vessels are not located in buildings. The external environment may require suitable controls to avoid potential contamination wherever the excipient or in-process material is exposed.

Excipient Properties and Functionality: Excipients are frequently used in those types of drug products for which physical characteristics, such as particle size, may be important. Although the manufacturer of the finished dosage form is primarily responsible for identifying the particular physical characteristics needed, it is also the responsibility of the excipient manufacturer to control excipient manufacturing processes to ensure consistent conformity to excipient specifications. Wherever possible, consideration should be given to the end use of the excipient. This is particularly important if the excipient is a direct component of a sterile drug product or one that is claimed to be pyrogen-free.

Consistency of Manufacture and Change Control: A thorough understanding of the manufacturing process and effective control of change can best ensure consistency of excipient quality from batch to batch. Implementation of changes may also have consequences for registration filings with regulatory agencies.

Changes in excipient manufacturing processes may result in changed physical or chemical properties of the excipient that are evident only during subsequent processing or in the finished dosage form. This is particularly important in the context of the pharmaceutical product approval process where bioequivalence comparisons are made between pivotal, clinical trial batch (*bio batch*) production and commercial scale-up batches. Changes made to the excipient supplied for the commercial product from the excipient supplied for the bio batch should not affect the quality and performance of the commercial drug product. Scale-up of excipients to commercial production may involve several stages, and data may be required to demonstrate consistency between batches through the scale-up process.

Traceability: Traceability of batch-related records to facilitate investigations and retrieval of product is also a key requirement of GMP.

APPLICATION OF GMP PRINCIPLES

It is the responsibility of the excipient manufacturer to designate and document the rationale for the point in the manufacturing process at which appropriate GMP are to be applied. From this point on, appropriate GMP should be applied. The manufacturer should apply a level of GMP to each manufacturing stage commensurate with the importance of that step in ensuring product integrity. This may be demonstrated by means of the use of a risk assessment procedure (for example, HACCP, FMEA).

The stringency of GMP in excipient production should increase as the process proceeds from early manufacturing to final stages, purification, and packaging. Physical processing (for example, granulation, coating, or physical manipulation of particle

size such as milling or micronizing) as well as chemical processing of excipients should be conducted at least to the standards suggested by this chapter.

It should be recognized that not all intermediates may require testing. An excipient manufacturer should, however, be able to identify critical or key points in the manufacturing process where selective intermediate sampling and testing are necessary in order to monitor process performance.

GENERAL AUDITING CONSIDERATIONS

Audits of an excipient operation will be influenced by the purpose of the audit and the intended use of the excipient. The key stages of a production process should be examined to determine whether the manufacturer controls these steps so that the process performs consistently. Overall, an audit should assess the excipient manufacturer's capability to deliver a product that consistently meets established specifications.

The audit team may consist of engineers, laboratory analysts, purchasing agents, computer experts, maintenance staff, and other personnel as appropriate to the scope and purpose of the audit. External auditors must respect the confidentiality of the manufacturer's processes and other disclosures.

An audit should focus on the quality-critical processing steps that are necessary for producing an excipient that meets established physical and chemical criteria. These steps should be identified and controlled by the excipient manufacturer. Quality-critical processing steps can involve a number of unit operations or unit processes. Quality-critical steps can include, but are not limited to, the following:

- phase changes involving the desired molecule, solvent, inert carrier or vehicle (for example, dissolution, crystallization, evaporation, drying, sublimation, distillation, or absorption),
- phase separation (for example, filtration or centrifugation),
- chemical changes involving the desired molecule (for example, removal or addition of water of hydration, acetylation or formation of a salt),
- adjustments of the solution containing the molecule (for example, pH adjustment),
- precise measurement of added excipient components, in-process solutions, and recycled materials (for example, weighing or volumetric measurements),
- mixing of multiple components,
- changes that occur in surface area, particle size, or batch uniformity (for example, milling, agglomeration, or blending).

AUDIT CHECK POINTS

A good approach for an excipient plant audit is a review of the following areas:

- nonconformities—such as the rejection of a batch that did not meet specifications, customer complaints, return of a product by a customer, or retrieval of a product. The manufacturer should have determined the cause of the nonconformity, prepared a report of the investigation, and initiated and documented subsequent corrective action. Records and documents should be reviewed to ensure that nonconformities are not the result of a poorly developed or inconsistent process;
- customer complaint files—such as reports that some aspect of the product is not entirely suitable for use, because such problems may be caused by impurities or inconsistencies in the excipient manufacturing process;
- change control logs—to ascertain whether the company evaluates its significant changes to decide whether the customer and/or regulatory authority should be notified;
- nonconforming products meeting or Material Review Board documents and/or equivalent records that demonstrate that the disposition of nonconforming product is handled in an appropriate manner by responsible individuals;
- master formula and production records for frequent revisions that may reveal problems in the excipient production process;
- evidence for the presence of unreacted intermediates and solvent residues in the finished excipient;
- materials management systems, to ensure adequate control over nonconforming materials so that they cannot be sold to customers or used in manufacturing without authorization;
- review of a process flow diagram, to aid understanding of the various processing stages. The critical stages and sampling points should be identified as part of the review of the processing records;
- review of contamination control measures.

In evaluating the adequacy of measures taken to prevent contamination and cross-contamination of materials in the process, it is appropriate to consider the following risk factors:

- the type of system (for example, open or closed). Enclosed systems in chemical plants often are not closed when they are being charged and/or when the final product is being emptied. In addition, the same reaction vessels are sometimes used for different reactions;
- the form of the material (for example, wet or dry);
- the stage of processing and use of the equipment and/or area (for example, multipurpose or dedicated);
- continuous versus batch production.

General Chapters

DOCUMENTATION AND RECORD REVIEW

Documentation required for the early steps in the process need not be as comprehensive as in the latter stages of the process. It is important that a chain of documentation exist and that it be complete when the following is the case:

- the excipient can be identified and quantified for processes where the molecule is produced during the course of the process. For batch production, a theoretical mass balance may also be established with appropriate limits, because deviations from tolerance are a good indicator of a loss of control;
- an impurity or other substance likely to adversely affect the impurity profile or form of the molecule is identified, and subsequent attempts are made to remove it.

As chemical processing proceeds, a chain of documentation should be established that includes the following:

- a documented process,
- the identification of critical processing steps,
- appropriate production records,
- records of initial and subsequent batch numbers,
- records of raw materials used,
- comparison of test results against meaningful standards.

If significant deviations from the normal manufacturing process are recorded, there should be evidence of suitable investigations and a review of the quality of the excipient. Complete documentation should be continued throughout the remainder of the process for quality-critical processing steps until the excipient is packaged and delivered to the end user. The batch should be homogeneous within the manufacturer's specifications. This does not necessitate the final blending of continuous-process material if process controls can demonstrate compliance with specifications throughout the batch.

In order to promote uniformity in excipient GMP inspections, the following basic requirements should be established:

- assignment of a unique batch number to the excipient, enabling it to be traced through manufacture to release and certification,
- suitable controls for the preparation of a batch record for batch processing and/or a production record, log sheet, or other appropriate documentation for continuous processing,
- demonstration that the batch has been prepared using GMP guidelines from the processing point at which excipient GMP have been determined to apply,
- confirmation that the batch is not combined with material from other batches for the purpose of either hiding or diluting an adulterated batch,
- records showing that the batch has been sampled in accordance with a sampling plan that ensures a representative sample of the batch,
- records showing that the batch has been analyzed using scientifically established test methods designed to ensure that the product meets established standards, specifications, and characteristics,
- stability data adequate to support the intended period of use of the excipient; these data can be obtained from historical data, from actual studies on the specific excipient, or from applicable model product studies that can reasonably be expected to simulate the performance of the specific excipient.

⟨1079⟩ GOOD STORAGE AND DISTRIBUTION PRACTICES FOR DRUG PRODUCTS

INTRODUCTION

This general information chapter describes good storage and distribution practices to ensure that drug products (medicines) reach the end user (practitioners and patient/consumers) with quality intact.

In the context of this chapter, the following definitions are used.

SCOPE

Good storage and distribution practices apply to all organizations and individuals involved in any aspect of the storage and distribution of all drug products, including but not limited to the following:

- Manufacturers of drug products for human and veterinary use where manufacturing may involve operations at the application holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Packaging operations by the manufacturer or a designated contractor for the applicant holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer

General Chapters

- Laboratory operations at the manufacturer's or at the contractor's site
- Physician and veterinary offices
- Pharmacies including but not limited to retail, compounding, specialty, mail order, hospital, and nursing home pharmacies
- Importers and exporters of Record
- Wholesale distributors; distribution companies involved in automobile, rail, sea, and air services
- Third-party logistics providers, freight forwarders, and consolidators
- Health care professional dispensing or administering the drug product to the end user
- Mail distributors including the U.S. Postal Service (USPS) and other shipping services including expedited shipping services

The information is intended to apply to all drug products regardless of environmental storage or distribution requirements.

It is recognized that conceivably there are special cases and many alternative means of fulfilling the intent of this chapter and that these means should be scientifically justified. Although this chapter is not intended to address the storage and distribution of active pharmaceutical ingredients (APIs), excipients, radioactive products, reagents, solvents, medical devices, medical gases, or clinical trial materials for which storage requirements may not yet be defined (e.g., Phase I clinical trial drug products), the general principles outlined here may be useful if applied selectively or comprehensively.

This general information chapter does not supersede or supplant any applicable national, federal, and/or state storage and distribution requirements, or USP monographs. General Chapter ⟨659⟩ *Packaging and Storage Requirements* contains definitions for storage conditions. This chapter is not intended to cover counterfeiting, falsified medicines, drug pedigrees, or other supply chain security, including chain of custody issues.

General Chapters

BACKGROUND INFORMATION

Storage and distribution processes may involve a complex movement of product around the world, differences in documentation and handling requirements, and communication among various entities in the supply chain. The translation of best practices into good storage and distribution meets these challenges and sets forth a state of control.

The good storage and distribution practices described in this chapter should facilitate the movement of drug products throughout a supply chain that is controlled, measured, and analyzed for continuous improvements and should maintain the integrity of the drug product in its packaging during storage and distribution.

RESPONSIBILITIES

The holder of the drug product application, the drug product manufacturer (in the case of many OTCs, where there is no application) and the repackager bear primary responsibility and accountability including but not limited to the following:

- The decision for regulatory submissions, where applicable, relative to the contents of this chapter for the storage and distribution of drug products. If breaches occur in any of the QMS systems and cannot be justified or documented with scientific evidence, the appropriate entity should consider action with the product to ensure the public safety.
- Determining proper storage and handling practices
- Communicating storage and distribution practices through the supply chain
- Drug product stability profiles or the associated stability information from the holder, inclusive of distribution conditions and excursions that may be allowable should they occur. These stability profiles include the approved storage conditions for the shelf life of the drug product and, where appropriate, supporting data for the distribution conditions, if these differ from the storage conditions.
- Appropriate firms, such as an applicant holder, are to convey relevant environmental requirements (e.g., when appropriate, product-specific lifecycle stability data), when needed to support deviations or temperature excursions. If stability data cannot be reviewed or is not shared, an assessment may be needed to consider regulatory review or other appropriate actions (e.g., destruction of product or additional stability testing).
- Recalling the drug product if it is found to be adulterated in any part of the supply chain

However, all organizations along the supply chain bear responsibility for ensuring that they handle drug products within adequate storage and distribution parameters that will not affect the drug product identity, strength, quality, purity, or safety.

Each holder of drug product is responsible and accountable for the receipt from an entity and transfer out of the drug product to the next entity.

LABELING CONSIDERATIONS FOR DRUG PRODUCTS

The environmental requirements for drug product storage conditions should be indicated on the drug product primary container–closure system. If space on the immediate container is too small (e.g., an ampule) or is impractical for the container–closure system (e.g., blister package), this information can be placed on the most immediate container of appropriate size (e.g., carton). Environmental storage conditions and/or environmental warning statements should be evident, securely fixed, and indelible on the outermost container (generally the shipping container).

Products classified as hazardous materials and/or dangerous goods by the U.S. Department of Transportation or other relevant authorities or bodies should be labeled, stored, and handled in accordance with applicable federal/state/local regulations. Drug products classified as controlled substances by the U.S. Drug Enforcement Administration or by individual state requirements should be labeled and handled in accordance with applicable regulations.

Good practices and controls for labeling should provide the receiver with instructions for the correct handling of the drug product upon receipt. When a drug product's storage conditions are not readily available, use the storage conditions described in USP's *General Notices and Requirements* or the applicable USP monograph; or, contact the drug manufacturer for further information.

Product labels with expanded information beyond the single long-term storage temperature ensure ease of transport and use for shippers, distributors, healthcare professionals, and patients. Product labels should clearly define the storage temperature range, and broader distribution or in-use temperature ranges where allowable. Products labeled "Keep in a cold place" or "Do not freeze" are subject to interpretation and are discouraged if used without accompanying temperature ranges. USP storage definitions and temperature ranges are defined in *General Notices and Requirements*.

During international transport, the proper language(s) should be used to ensure that handlers understand the requirements set forth on drug product labeling. The use of symbols that are recognized by international organizations is advisable.

Drug products can be transported at temperatures outside of their labeled storage temperatures if stability data and relevant scientific justification demonstrate that product quality is maintained. The length of the stability studies and the storage conditions for a drug product should be sufficient to cover the shipment, distribution, and subsequent use of the drug product. The data gathered from ICH, Q1A R2, accelerated testing or from testing at an ICH intermediate condition may be used to evaluate the effect of short-term excursions outside of the label storage conditions that might occur during storage and/or distribution.

General Chapters

Change to read:

QUALITY MANAGEMENT SYSTEM

Good storage and distribution practices require that entities involved in the storage and/or distribution of drug products maintain a Quality Management System (QMS) that is based on standard quality concepts, includes good manufacturing practice (GMP) in compliance with the appropriate regulatory agency(s), and is complementary to the ICH quality guidances, including ICH *Q10 Pharmaceutical Quality System* and ICH *Q9 Quality Risk Management*. In the context of this chapter, the QMS includes the following management system programs: (1) *Storage Management System*, (2) *Distribution Management System*, (3) *Environmental Management System*, and (4) *Risk Management System*.

The storage and distribution QMS should, at minimum, cover the following elements: corrective and preventive actions (CAPA), change management, deviation/investigation management, and the management review process.

Written agreements (e.g., Quality Agreement, Technical Agreement, Service Level Agreements) should be in place between applicable organizations involved in the drug product supply chain. This means that the originating manufacturer may not be required to hold a Written Agreement with all parties in the supply chain. The use of written agreements ensures clarity and transparency, and delineates the responsibilities of each organization in the supply chain.

Good Documentation Practices

Good documentation practices should be practiced in the QMS. This documentation includes standard operating procedures and corporate policies and standards, as well as protocols and other written documents that delineate the elements of the QMS. The QMS programs should describe events and actions that must be documented as well as the proper verbiage to be used, the copies required, and any other items that will ensure adequate processing of the drug product and prevent delays. The documentation process should use a standard such as a quality manual or other practice and, should include routine assessment for review and update as needed.

Written procedures should ensure that drug products are held in accordance with their labeling instructions and associated regulatory requirements. Procedures should provide the written steps needed to complete a process and ensure consistency and standard outcomes. The following elements should be included: (1) how and when a product should be moved from one transport container/vehicle into another, (2) how products are handled when equipment malfunctions or when there are delays in distribution due to Customs hold, and (3) how to communicate with the necessary parties.

The QMS should require monitoring of processes to demonstrate that a state of control is being maintained, where the set of controls consistently provides assurance of continued process performance and product quality (ICH Q10).

If deviations occur, a nonconformance should be documented, and investigation should be performed and documented as appropriate. The investigative process should determine the root cause(s) of the deviation. For example, the following should be determined: whether the drug product experienced stress, damage, delays, or environmental lapses, or whether there were errors in documentation. The associated supply quality management staff should have final responsibility for approving or rejecting the investigation. The investigation process should be linked to the risk management program to ensure that proper mitigation occurs and preventive measures are put in place.

For example, a written investigation should be performed if the receiving and/or transferring processes result in a drug product being subjected to unacceptable temperature conditions or contamination (e.g., pests, microorganisms, or moisture). Any breach of standard operating procedures should be documented with a risk justification as needed. This information should be forwarded to the appropriate organization responsible for the drug product. The drug product should be quarantined, and final disposition should be based on good science with appropriate evidence to justify the decision(s).

Manufacturers should develop written procedures for recording the security process that confirms container–closure integrity for drug products that require special handling, such as security seals for controlled substances. Returned and salvaged goods records should address how the drug product is assessed through a written procedure. In addition, training on such procedures should be part of the QMS.

Records should be retained for purchases and sales of drug products and should show the date of purchase or supply; the name of the drug product and the amount; the name and address of the supplier or consignee; and the associated lot numbers. These records should allow for the traceability of a drug product in the supply chain.

All records and documents should be maintained in accordance with a traceable records-retention program and should be made available upon request to regulatory agencies. These documents should be approved, signed, and dated by the department responsible for the QMS.

Storage Management System

STORAGE LOCATIONS AND PROCESSES

It is important that each entity define their appropriate storage locations to ensure that adequate controls are in place. These locations include buildings and facilities for drug product storage (e.g., warehouse, storage or hold area, the original manufacturer's warehouses, contractor warehouses, wholesale distribution warehouses, mail order or retail pharmacy storage area, hospital or nursing home pharmacy storage areas; and border Customs storage areas).

In these locations, two basic processes can occur. First, receiving for storage is the act of bringing a drug product into a facility, while transferring refers to the moving of a drug product internally within a facility or into or out of a vehicle. Second, storing and holding refers to the act of maintaining temporary possession of a drug product in the supply chain process, during which no movement of the product will occur.

STORAGE IN BUILDINGS AND FACILITIES

Drug product storage areas are required to maintain the product temperature between the limits as defined on the product label. Buildings and facilities used for the warehousing, storage, and/or holding of drug products should be of adequate size for their intended use. These facilities should be adequate to prevent overcrowding. The building and facility should be designed to control environmental conditions where necessary and should be made of readily or easily cleanable materials. Sanitation and pest control procedures should be written, indicating frequency of cleaning and the materials and methods used. The pest-control program should ensure the prevention of contamination as well as the safe use of pesticides. Records of all cleaning and pest-control activities should be maintained.

Storage should be orderly and should provide for the segregation of approved, quarantined, rejected, returned, or recalled drug product. If computerized systems are used for the control of storage conditions, the software should be appropriately qualified for its intended purposes. Facilities should have controls that mitigate risks such as fire, water, or explosion. Certain drug products may cause these risks and should be stored accordingly. Storage areas, when not computerized, should be appropriately visually labeled.

Storage facilities themselves, unless thermostatically controlled, cannot be validated; however, they can be qualified via a mapping process. The generator back-up power supply should be qualified.

RECEIVING AND TRANSFERRING DRUG PRODUCTS

Storage of a drug product includes not only the period during which the drug product is held in the manufacturer's storage areas but also time spent at the receiving bay area. When drug products arrive at warehouse loading docks and other arrival areas, they should be transferred as quickly as possible to a designated storage or within a time period that is consistent with the risk and exposure of the product in the receiving area to a designated storage environment to ensure minimal time outside specified storage conditions as described in a written procedure.

Relative to the incoming receipt of drug product, it is recognized that the process of product reaction to ambient conditions begins immediately and may occur quickly (e.g., reach temperature equilibrium within minutes to a few hours depending on details such as the product mass, volume, and packaging density taking into account secondary and tertiary packaging)[1]. Time spent in a transport vehicle is considered to be part of the distribution process and is not a storage location.

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[1] JP Emond, *Study for Temperature Sensitive Product: Preliminary Testing*, October 2009, University of Florida.

Receiving docks should protect drug product deliveries from inclement weather during unloading. Any storage area, including loading and unloading docks for receipt and distribution of drug products, should be clean, cleanable, and free from pests. The incoming receiving area should limit access to authorized persons. Where appropriate, the delivery vehicle/container should be examined before unloading to ensure that adequate protection from contamination was maintained during transit. Deliveries should be examined at receipt in order to check that containers are not damaged and that the consignment corresponds to the order. The results of this examination should be documented.

Areas should be designated to provide an adequate space in which containers of drug products can be cleaned and opened for sampling. If sampling is performed in the receiving area, it should be done in a manner that prevents contamination and cross-contamination and ensures that environmental requirements for the drug product are not breached.

Adequate precautions should be taken to prevent theft and diversion of drug products. Drug products that have been identified as counterfeit should be quarantined to prevent further distribution. The appropriate regulatory agencies should be contacted according to established procedures.

Appropriate delivery records (e.g., as applicable, transport vehicle movement papers, receiving/delivery records, data logging records, temperature recorders and similar devices, bill of lading, house air waybill, master air waybill, etc.) should be reviewed by each receiving entity in the supply chain to determine if the product has been subjected to any transportation delays or other events that could have exposed the product to undesirable conditions. Each entity should ensure that their respective Service Level Agreement documents and supporting documents such as SOPs cover delivery and receiving responsibilities of the transactional parties.

Smoking, eating, and drinking should not be permitted in any storage/hold areas.

REFRIGERATORS AND FREEZERS

Refrigerators and freezers used to store drug products are required to maintain the product temperature between the limits as defined on the product label. Typically, a refrigeration unit specification would be set to 5° with an allowable range of ±3° to store products labeled 2°–8°. Freezer temperatures may vary and typically range from −25° to −10°. Some frozen drug products, however, require lower temperatures, e.g., dry ice or liquid nitrogen temperatures.

Regular operating procedures and maintenance protocols should be in place along with written contractual agreements for all maintenance and evaluation procedures including the following:

1. Items should be stored in the units in a manner that allows adequate air flow to maintain the specified conditions.
2. Units should be positioned in the facility so that they are not subjected to environmental extremes that could affect their performance. If this cannot be prevented, the mapping protocol should include a provision for testing during the anticipated environmental extremes.
3. Large commercial units such as walk-in cold rooms are qualified via a temperature mapping study or other type of qualification process to determine the unit's suitability for storing drug products. A suitable number of temperature-recording devices should be utilized to record temperatures and to provide temperature area maps. Thereafter, the units should be monitored as determined by the results of the mapping study. Refer to the *Temperature Monitoring* section under *Environmental Management System*.
4. Units should utilize recording systems to log and track temperatures. Alarm systems should be an integral part of the monitoring system for both refrigerators and freezers. While automated systems monitor units continuously, manual checks should be performed as appropriate to the validation program. When automated systems are not available, manual systems may be used.

Distribution Management System

Distribution of drug products occurs within a facility or location such as a manufacturer, wholesaler, pharmacy dispensing area, retail site, clinic/hospital/nursing home pharmacy, and the physician's practice. Distribution of drug products occurs as point-to-point movement within the supply chain between distribution facilities via semitrailer trucks, vans, emergency medical service vehicles, industry representatives' automobiles, trains, aircraft, sea vessels, and mail delivery vehicles.

Communication within the supply chain should be coordinated to determine proper timing for drug products to be transported and received, taking into account holiday schedules, weekends, or other forms of interruption. When international distribution is required, alerts should be made in advance and proper language should be used to ensure understanding of the requirements set forth on drug product labeling.

PACKAGING FOR THE DISTRIBUTION AND TRANSPORTATION PROCESSES

Pharmaceutical manufacturers should consider primary, secondary, and tertiary packaging that best protects the drug product during storage and distribution. Package performance testing should be documented as part of a manufacturer's QMS. Several standard test procedures are available for evaluating package performance for factors such as shock, vibration, pressure, compression, and other transit events. Organizations with standard test methods include the following: the American Society for Testing and Materials (ASTM) *Standard Practice for Performance Testing of Shipping Containers and Systems*, and the

International Safe Transit Association (ISTA) specifications for various types of transit modes such as less-than-truckload, small package, rail car, and air freight.

It is important to be aware that removal or modification of the original packaging may subject the product to unacceptable conditions.

The packaging (tertiary or thereafter) for the distribution of the drug product should be selected and tested to ensure that product quality is maintained and to protect the contents from the rigors of distribution including environmental or physical damage.

All drug products have storage requirements that may contain specific controls. The container used for transporting the drug product should be qualified on the basis of the labeled conditions of the product as well as anticipated environmental conditions. Consideration should be made for seasonal temperature differences, transportation between hemispheres, and the routes and modes of transport.

The type, size, location, and amount of the temperature stabilizers required to protect the product should be based on documented studies of specific distribution environments including domestic and international lanes, mode(s) of transport, duration, temperature, and other potential environmental exposures or sensitivities that may impact product quality. Transportation container materials such as warm/cold packs and materials used to control temperature conditions should be properly conditioned before use. Barrier protection may be important in helping to determine the position of materials such as gel packs in order to avoid direct contact with the drug product. It should be determined if studies are required to ensure that the dry ice and its vapors do not adversely affect the drug product, including the drug product labeling.

VALIDATION AND THERMAL PERFORMANCE QUALIFICATION FOR TRANSPORT SYSTEMS

Drug product transport systems should be continuously monitored by calibrated monitoring systems, (continuous verification), or shipping systems should be qualified and based on historical data relative to the process. However, it may be acceptable to use product stability data and supply chain risk assessment to justify shipping without either continuous monitoring or qualification of the shipping system.

Operational and performance shipping studies should on a generic level be part of a formal qualification protocol that may use controlled environments or actual field testing, depending on the projected transport channel. These studies should reflect actual load configurations, conditions, and expected environmental extremes. Testing should be performed on both active and passive thermal packaging systems.

Environmental Management System

While storage and distribution temperature ranges for drug products are labeled on the packaging, relative humidity effects occur over a much longer time frame. The primary container is designed and tested to protect the product from moisture; therefore, humidity monitoring should be considered when a product will be stored in an uncontrolled facility.

TEMPERATURE MONITORING

Environmental conditions are important parameters to consider in the storage and distribution of all drug products and may require monitoring depending on the requirements. When specific storage conditions are required and transportation qualification has not been performed, and in the absence of active or passive containers, environmental recorders or devices should be used to confirm that an acceptable range has been properly maintained during each stage in the supply chain.

Temperature is one of the most important conditions to control, and requirements for each drug product should be based on stability data. Temperatures should be tracked using a monitoring system, and the monitoring devices used should be included in a calibration and/or preventive maintenance program. Environmental monitoring devices should be calibrated for their range of operation. The monitoring devices used should provide an alert mechanism if the preset ranges are breached. The following practices and controls are examples of appropriate measures that should be put in place to ensure environmental control (see also *Monitoring Devices—Time, Temperature, and Humidity* ⟨1118⟩):

- Temperature-monitoring equipment, a monitoring device, a temperature data logger, or other such device that is suitable for its intended purpose should be used.
- An appropriate number of temperature monitors or some other form of recordation or proof of temperature control. Temperature monitor(s) should be used with every distribution process unless another process has been put in place to ensure specified temperature ranges.
- Electronic temperature monitors should be calibrated to National Institute of Standards and Technology (NIST) or other suitable standard.
- Chemical temperature indicators may be used as appropriate.
- Predetermined temperature ranges should be set for all applicable areas, as well as a plan of action in the event of an unacceptable excursion.

TEMPERATURE MAPPING

The basis of any temperature mapping in a temperature controlled space (e.g., facility, vehicle, shipping containers, refrigerator, freezer) is the identification and documentation of a sound rationale used for a given mapping procedure. The temperature variability associated with mapped locations and the level of thermal risk to the product should be defined, unless another process has been put in place to ensure environmental control.

A temperature mapping study should be designed to assess temperature uniformity and stability over time and across a three-dimensional space. Completing a three-dimensional temperature profile should be achieved by measuring points at not less than three dimensional planes in each direction/axis—top-to-bottom, left-to-right, front-to-back, where product will be present.

When temperature mapping is necessary, it should begin with an inspection of the facility, equipment and/or vehicle and should be re-evaluated as appropriate. Environmental mapping also should be performed after any significant modification to the distribution system that could affect drug product temperature.

Facility temperature mapping: The following factors, which may contribute to temperature variability, should be considered during the process of temperature mapping storage locations: (1) size of the space; (2) location of HVAC equipment, space heaters, and air conditioners; (3) sun-facing walls; (4) low ceilings or roofs; (5) geographic location of the area being mapped; (6) airflow inside the storage location; (7) temperature variability outside the storage location; (8) workflow variation and movement of equipment (weekday vs. weekend); (9) loading or storage patterns of product; (10) equipment capabilities (e.g., defrost mode, cycle mode); and (11) SOPs.

The recording of temperatures during the thermal mapping of a warehouse or cold room should be sufficient in time frame to capture workflow variation that may impact air flow and the resulting temperature fluctuation (i.e., a period of one week is recommended for data collection and should capture workflow cycles).

Equipment (container/trailer) temperature mapping: To minimize risk of product exposure to damaging temperatures during transport, dedicated containers/vehicles cargo space should be mapped. When complete fleet mapping (i.e., wholesaler or distributor vehicles) is not realistic or appropriate, minimally at least one container/vehicle from the fleet must be mapped. Thereafter, the following conditions should be considered: (1) SOPs, including loading and unloading procedures; (2) route-specific operation of the temperature control equipment; (3) seasonal effects encountered on expected routes; (4) loading patterns; and (5) transport durations.

When nondedicated (i.e., mail carriers) transport containers/vehicles and equipment are used, they should be designed to minimize the risk of contamination of the product being handled. If environmental mapping of such vehicles is not performed, some other means of control should be in place to ensure that the drug product is adequately protected. Mapping by the shipper may not be necessary if the shipper uses a transport container that is properly insulated and has been previously qualified for the duration of the distribution process by the transport container manufacturer via a mapping study or if drug products are continuously monitored by calibrated monitoring systems (continuous verification).

The vehicle in which drug products are transported should be mapped to determine the appropriate placement of temperature-recording devices and to confirm that the load configuration is not restricting air flow. The following are recommended practices and controls for vehicles that receive and transfer drug products:

1. Transport containers/vehicles and equipment used to store and transport drug products should be suitable for their intended function.
2. Procedures should be established that describe how to operate, clean, and maintain transport containers/vehicles and equipment used in the storage and distribution of drug products.
3. Transport containers/vehicles should be designed to prevent damage to the drug product, and pharmaceutical manufacturers should collaborate with their transporter to determine contingency response plans for how drug products are handled when equipment malfunction.
4. When drug product must be moved from one transport container/vehicle into another, the proper load configuration should be followed.
5. It should be understood how communication is made to the necessary entities when such transfer occurs.
6. Subcontracted vehicles should be considered in contractual agreements and audits, and documentation should be maintained for their use.

Temperature mapping should account for maximum and minimum loads to capture temperature variability resulting from variations in temperature mass of the payload. Performance of equipment under extreme scenarios including door open, door closed, and simulated equipment failure should be taken into account.

Thermal mapping of vehicles should be representative of the fleet with the intention of capturing variability across the range of vehicles (type of vehicle including non-refrigerated equipment, use, heating and/or cooling system). A periodic requalification program should be documented.

Mapping for both facilities and transportation containers/vehicles should be done in a way that confirms their fitness for operation during periods of expected extreme weather (e.g., summer and winter). Facilities should be mapped under varying operating conditions—ideally during periods of greater variability, accounting for and capturing the result of any seasonal fluctuations of inventory movement, equipment movement, or workflow variation.

The temperature-mapping protocol and associated number of temperature data loggers used to map a three dimensional space should meet the intent of demonstrating three-dimensional uniformity and compliance with product requirements. For

both facility and trailer/container temperature mapping, the ambient conditions should be recorded and correlations between ambient conditions and potential thermal risks inside the controlled space should be identified. Drug products should not be stored in areas where a thermal risk has been identified as a result of the temperature mapping. Areas identified as being unsuitable for storage should be clearly labeled as such to ensure that they are not used.

Temperature data loggers should be used for temperature mapping and PQ testing of facilities, equipment, and transportation containers used for storage or transportation of temperature-sensitive medicinal products. Temperature data loggers and any associated software applications should be appropriately validated. Certificates of calibration to an NIST or other international traceable standard should be available for individual monitoring devices.

EXCURSIONS

The mapping process will help determine when excursions could occur and are useful when pharmaceutical manufacturers develop a plan for dealing with them. Alarms should be used to reveal environmental excursions during operations. Temperature excursions for brief periods outside of respective storage label conditions may be acceptable provided stability data and scientific/technical justification exists demonstrating that product quality is not affected (see Health Canada's GUI 0069 entitled, *Guidelines for Temperature Control of Drug Products During Storage and Transportation,* 2011).

MEAN KINETIC TEMPERATURE (MKT) CALCULATION

The MKT is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT may be considered as an isothermal storage temperature that simulates the non-isothermal effects of storage temperature variation. It is not a simple arithmetic mean.

The temperatures used for calculating MKT can be conveniently collected using electronic devices that measure temperatures at frequent intervals (e.g., every 15 minutes). MKT can be calculated directly or the data can be downloaded to a computer for processing. Software to compute the MKT is available commercially.

For dispensing sites, such as pharmacies and hospitals, where the use of such instruments may not be feasible, devices such as high-low thermometers capable of indicating weekly high and low temperatures may be employed. The arithmetic mean of the weekly high and low temperatures is then used in the calculation of MKT. MKT is calculated by the following equation (derived from the Arrhenius equation):

$$T_k = \frac{\Delta H/R}{-ln\left(\frac{e^{-\Delta H/RT_1}+e^{-\Delta H/RT_2}+...+e^{-\Delta H/RT_n}}{n}\right)}$$

where T_k is the mean kinetic temperature; ΔH is the heat of activation, 83.144 kJ · mole^{-1} (unless more accurate information is available from experimental studies); R is the universal gas constant, 8.3144×10^{-3} kJ · mole^{-1} · degree^{-1}; T_1 is the value for the temperature recorded during the first time period, e.g., the first week; T_2 is the value for the temperature recorded during the second time period, e.g., second week; and T_n is the value for the temperature recorded during the nth time period, e.g., nth week, n being the total number of storage temperatures recorded during the observation period. [NOTE—All temperatures, T, are absolute temperatures in degrees Kelvin (K).]

MKT DURING STORAGE AND DISTRIBUTION

The holding of a drug may occur as part of storage and distribution practices. Drug products in the distribution supply chain may be held at temperatures outside their labeled storage requirements as determined by an appropriate stability study. Drug products stored either in warehouse conditions or in transportation modes may experience excursions from their acceptable temperature ranges. Each product excursion must be evaluated to determine the final product effect. The means of evaluation must be scientifically sound with documented technical justification that the integrity of the drug product has not been affected. One method of analysis for drug product stored outside its respective label storage conditions is the use of an MKT calculation.

Because MKT expresses the cumulative thermal stress a drug product experiences, it is considered an acceptable practice for storage, and it follows that it should be considered for transit excursions in the process of distribution. The calculation must be justified for use with distribution excursions by confirming that the stability limiting characteristic of the product follows first order kinetics over the temperature range encountered. The ICH stability-testing guidelines define MKT as a "single" derived temperature, which, if maintained over a defined period, would afford the same thermal challenge to a pharmaceutical product as would have been experienced over a range of both higher and lower temperatures for an equivalent defined period.

The MKT analysis must be based on good science and should take into account the integrity of the product. The calculated MKT is not sensitive to the impact of excursions that may occur if the baseline is a long period of time such as a storage segment or the entire lifetime of the drug product. For shorter baseline periods of time, such as transport segments, an excursion

can have a significant impact on the resulting MKT for that segment; however, this would not necessarily have a significant impact on product quality.

The MKT analysis may be used for storage conditions that have exceeded the acceptable parameters for a drug product, for a short period of time and is not intended to be a measure for long-term storage.

Knowing the MKT for an excursion is useful for evaluating the potential impact on product quality. However, it is also essential to know the upper and lower temperature limits of any excursion. If these extreme temperatures are outside available stability data, it may not be possible to predict the quality impact of the excursion with any confidence regardless of the MKT. Although higher temperatures are given greater weight in the calculation, the calculation of MKT for nonfrozen product that becomes frozen for any amount of time may not result in an acceptable temperature although the product may not be adulterated. At higher temperatures the kinetics of degradation may change or new degradation reactions may occur; at lower temperatures (near freezing) a phase change may occur that is known to have a negative impact on the quality of some drug products (e.g., some proteins and vaccines). For an example of a calculation, see ●*Pharmaceutical Calculations in Pharmacy Practice* ⟨1160⟩● (CN 1-May-2017).

Emergency Medical Service Vehicles, Automobiles, and Van Transportation

Road vehicles used to transport drug products (e.g., ambulances and other emergency response vehicles, vans, or automobiles, including those used by sales representatives to transport physicians' samples) should be suitable for their purpose. Monitoring devices should be placed in different areas of the trunk or cabin where the drug product will be positioned during seasonal extremes (e.g., summer and winter). The monitor should be secured so that it is immobile, and there should be no ambiguity about its exact position within the payload so that the monitor is always placed in the same position. Monitoring devices used on or in packages or on containers may also be used. Suitable measures should be taken to maintain the drug product within the allowable limits of the labeled storage requirements. Storage of physician drug product samples by sales representatives is regulated under 21 CFR Part 203.34(b)(4).

Mail Order Pharmacy Distribution

The mailing party is accountable for the appropriate mailing process. Mail distributors including the U.S. Postal Service (USPS) and other shipping services including expedited shipping services are responsible to provide the service contracted.

In the event that the package cannot be delivered as scheduled, the package should be returned to the mailing pharmacy.

Risk Management System

Risk Management System strategies should ensure that each organization's best interests are served by adhering to proper practices, controls, and procedures, including but not limited to the following: the nature of the drug products; distribution requirements on the readable container labeling; exposure to adverse environmental conditions; number of stages/receipts in the supply chain; manufacturer's written instructions; contractors; and drugs at risk from freezing (vaccines, insulin, and biological products) or elevated temperatures (fatty-based suppositories, vaccines, insulin, and biological products).

Examples of risks include the following: (1) vibration that can cause aggregation of some drug products such as proteins and peptide-based drugs; (2) temperature excursions that may lead to phase changes (melting or freezing); (3) loss of container–closure integrity in transit that could cause glass fractures or loss of sterility in sterile drug product containers; and (4) ingress of water or oxygen that could lead to an increase in degradation products. Appropriate firms such as applicant holders are recommended to convey relevant environmental requirements when needed to support deviations or excursions. There may be alternate ways of determining acceptable environmental conditions and these should be documented and justified.

Pharmaceutical manufacturers should ensure that suppliers of drug product transportation are monitored. Auditing transportation firms should be carried out routinely to ensure adequate product handling. The manufacturer's change control system should capture and evaluate changes in logistic factors such as warehouse or receiving areas and vehicle changes.

CONCLUSION

The practices and processes set forth in this general information chapter apply to storage and distribution as part of the lifecycle management of drug products. All involved should ensure the product to its point of use, creating a contiguous supply network that is collaborative and emphasizes preventive measures to protect drug product quality. The increase in global processes coupled with products requiring special environmental controls highlights the need for a strong QM program. QM should provide the foundation for maintaining the storage and distribution practices in a continual improvement program and part of an overall management system review by each entity, as appropriate, in the supply chain.

It is equally important to stay current and be ready to change as new solutions evolve. These new technologies should be considered in developing strategies for good distribution practices, controls, and procedures.

GLOSSARY

Adulteration: FDA FDC Act, SEC. 501 (351), A drug or device shall be deemed to be adulterated, if (2)(A) It has been prepared, packed, or held under insanitary conditions it may have been contaminated with filth, or whereby it may have been rendered injurious to health; or (B) the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice to assure that such drug meets the requirements of this Act as to safety and has the identify and strength, and meets the quality and purity characteristics, which it purports or is represented to possess.

Continuous improvement: Recurring activity to increase the ability to fulfill requirements (see *Quality Management Systems—Fundamentals and Vocabulary. ISO Standard 9000:2005*).

Distribution: Refers to elements such as shipping and transportation activities that are associated with the movement and supply of drug products.

Distribution management system: A program that covers the movement, including storage and transportation, of drug products.

Documentation: Recorded information.

Drug products: Medicines, including marketed human and veterinary prescription finished dosage medications, in-process/intermediate/bulk materials, drug product samples, clinical trial materials, over-the-counter products (OTC).

End user: The patient as well as the healthcare provider administering the drug product to the patient.

Environmental management system: A management system that allows for the identification of quality critical environmental aspects (such as temperature, humidity, and/or other environmental factors) for the drug product and ensures that adequate processes to maintain that environment are in place.

Hazardous materials and/or dangerous goods: Any item or chemical which, when being transported or moved, is a risk to public safety or the environment, and is regulated as such under any of the following: Hazardous Materials Regulations (49 CFR 100–180); International Maritime Dangerous Goods Code; Dangerous Goods Regulations of the International Air Transport Association; Technical Instructions of the International Civil Aviation Organization; or the U.S. Air Force Joint Manual, *Preparing Hazardous Materials for Military Air Shipments*.

International Conference on Harmonization (ICH) Guidance for Industry, *Q10 Pharmaceutical Quality System; ICH Q9, Quality Risk Management; and, ICH Q1A R2, Stability Testing of New Drug Substances and Products*: Internationally harmonized documents intended to assist the pharmaceutical industry.

Mean kinetic temperature (MKT): The single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures.

Preventive actions: The measures to eliminate the cause of a potential nonconformity or other undesirable potential situation.

Quality: The physical, chemical, microbiological, biological, bioavailability, and stability attributes that a drug product should maintain in order to be deemed suitable for therapeutic or diagnostic use. In this chapter, the term is also understood to convey the properties of safety, identity, strength, quality, and purity.

Quality management system (QMS): In the context of this chapter, minimally a set of policies, processes, and procedures that enable the identification, measurement, control, and improvement of the distribution and storage of drug product. It is the management system used to direct and control a company with regard to quality (see ICH Q10 model and *Quality System—Fundamentals and Vocabulary, ISO Standard 9000:2005*).

Risk management system: A systematic process used to assess, control, communicate, and review risks to the quality of a drug product across the product lifecycle. Integral to an effective pharmaceutical quality system, it is a systematic and proactive approach to identifying, scientifically evaluating, and controlling potential risks to quality as described in ICH Q10. It facilitates continual improvement of process performance and product quality throughout the product lifecycle. ICH Q9 Quality Risk Management provides principles and examples of tools that can be applied to different aspects of pharmaceutical quality.

Written agreement or contract (commonly referred to as a Quality Agreement, Technical Agreement, Service Level Agreement, or other): A negotiated, documented agreement between the drug product owner and service provider that defines the common understanding about materials or service, quality specifications, responsibilities, guarantees, and communication mechanisms. It can be either legally binding or an information agreement. A Service Level Agreement may also specify the target and minimum level of performance, operation, or other service attributes.

Storage management system: A program that is used to control the storage of drug products.

Supply chain: The continuum of entities spanning the storage and distribution lifecycle of a product to the end user.

Temperature stabilizer: A material or combination of materials that stores and releases thermal energy used to maintain a specified temperature range within an active or passive packaging container or system (e.g., water-, chemical-, or oil-based phase change material, such as carbon dioxide solid/dry ice and liquid nitrogen).

Transport vehicles: Vehicles used in the supply chain including semitrailer trucks, vans, trains, airplanes, sea vessels, and mail delivery vehicles. Other vehicles, when used to transport drug products are included here, such as emergency medical service vehicles and industry representatives' automobiles.

⟨1080⟩ BULK PHARMACEUTICAL EXCIPIENTS—CERTIFICATE OF ANALYSIS

BACKGROUND

This general information chapter is derived from the *Certificate of Analysis Guide for Bulk Pharmaceutical Excipients*, prepared by The International Pharmaceutical Excipients Council of the Americas (IPEC-Americas), an international guidance document on the preparation and appropriate use of a Certificate of Analysis (COA) for these excipients, referenced throughout the chapter as "excipient(s)". The chapter defines the suggested elements of a Certificate of Analysis, provides a template for organizing required and optional data in a logical manner, and assists in establishing a uniform understanding of the roles and responsibilities of excipient manufacturers, distributors, and users.

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients intended for use in human drugs, veterinary drugs, and biologics. As an international guidance document, it cannot specify all national legal requirements nor cover in detail the particular characteristics of every excipient. When considering how to use this chapter, each manufacturer, distributor, or user should consider how it may apply to that specific manufacturer's product and processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes.

The chapter is divided into several parts. The first part provides background discussion necessary for the design and suggested elements of a COA. A template is provided to show the format and placement of information in the COA. This is followed by a detailed discussion to ensure that the purpose and meaning of the specific information contained in the COA is understood. For a list of terms used in this information chapter and their definitions, see *Appendix 1*.

GENERAL GUIDANCE

International regulations governing drugs require that components of the drugs be manufactured, processed, packed, and held in accordance with good manufacturing practices (GMPs). For a thorough discussion of GMPs that apply to excipient manufacture, see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩. The excipient is often a natural substance, mixture, or polymer whose chemical and physical properties are difficult to quantify and that is often used with a broad range of active pharmaceutical ingredients and in a diverse range of finished dosage forms. Until now, there were no guidance documents that specifically focused on the content or format of COAs for excipients and that addressed the diversity of both the excipients and their usage.

Preparation and Appropriate Use of a Certificate of Analysis—The Certificate of Analysis for excipients should be prepared and issued by the supplier of the material, following the general guidelines discussed below. Primary responsibility for the preparation of the COA belongs to the excipient manufacturer. It is most important that a complete and accurate COA be provided to the excipient user for specific lots or batches intended for use in the pharmaceutical industry. Additional considerations should be made for the preparation and issuance of a COA by a distributor of excipients.

The user of a bulk pharmaceutical excipient should always receive a COA for material to be used in the manufacture of a drug product. At a minimum, the user should perform adequate identification tests on each lot of excipient received before releasing it for use in the drug product. Specific identity tests should be used whenever possible. It is a regulatory requirement that excipients be assessed for conformity with all appropriate specifications. However, testing of all specification parameters may not be required for lot release if adequate compliance assurances are provided on the supplier's COA. Before using an excipient in a pharmaceutical product based on COA data, the user also should have an understanding of the supplier's control systems and compliance with GMPs, through appropriate auditing or qualification of the supplier.

Nevertheless, it is the responsibility of the user of the excipient to verify any of the analytical data contained in the COA if knowledge of such information is deemed essential to the use of that excipient. Such testing may go beyond the scope of the compendial methods described in the *NF*, or beyond those used to develop the information in the COA.

To use test results from a COA, the user must also establish the reliability of the supplier's COA test results by periodically performing all required tests and comparing the results obtained to the supplier's test results. Occasionally, it may not be possible to perform all the required tests because of special equipment requirements, etc., that may not be available to the user. Performing fewer than all these tests may be acceptable provided that the reliability of the supplier has been adequately determined using other appropriate supplier qualification techniques.

It is important to understand that these results may not always specifically correlate, especially when an excipient is produced as a continuous lot. However, the user's test results should demonstrate compliance with the specification requirement.

Use of Contract Facilities—Contract facilities are frequently used in the manufacture, testing, and distribution of excipients. When such facilities are used, the supplier of the excipient has the obligation to ensure that the facilities operate under appropriate quality standards (i.e., cGMP, GLP, etc.).

DESIGN AND SUGGESTED ELEMENTS OF A CERTIFICATE OF ANALYSIS

The suggested elements of a COA are listed below and are included in the following *Certificate of Analysis Template* section of this chapter. Excipient suppliers may organize the suggested elements presented in the COA template at their discretion; however, the parts of the template were designed to present the suggested and optional information in a logical manner. For a detailed description of each element and examples of statements, see the appropriate section below in this chapter.

The origin and the identity of the excipient are typically established in a *Header* section. The manufacturer and manufacturing site should be identified if different from the supplier and supplier location, enabling the user to make certain that the excipient comes from a qualified source. Although the manufacturer should be made known to the user, the use of codes for manufacturers and manufacturing sites on the COA to protect confidentiality is acceptable. The identity of the excipient must be definitively established by stating the compendial and trade name, the grade of the material, and applicable compendial designations.

A lot/batch number or other means of uniquely identifying the quantity of material covered by the COA and information relating specifically to it are typically included in a *Body* section. The lot number or other unique identification of the material, its date of manufacture, and product code or number should be stated and traceable to a specified lot. If applicable, the expiration date, recommended re-evaluation date, or other relevant statement regarding the stability of the excipient is typically included in this section. Any information required by the customer would also be included here.

The actual test results applicable to the quantity of material covered by the COA are included in an *Analysis* section. The test name, the result, the acceptance criteria or specifications, and a reference to the test method used should be included for each characteristic listed. Reporting of actual data and observations is recommended rather than nonspecific "passes" or "conforms" statements. If the reported results are derived from a skip-lot or reduced frequency testing program, or an average or in-process test result, this should be noted on the COA.

The *Certification and Compliance Statement* section is used to list various types of statements that may be required depending on the excipient and specific user needs. These statements are usually negotiated between supplier and user based on specific application requirements. Any declaration of the supplier that includes compliance of additional compendial or other regulatory requirements is typically included in this section.

Many excipients have applications other than pharmaceuticals, such as food, cosmetics, or industrial products. Any product listed as being in compliance with specific regulations should meet the specifications and requirements of that regulation and must be manufactured under appropriate GMPs.

The identity of the individual approving the content of the COA should appear on the COA. The page number and total number of pages should also appear on the COA. This information is usually included in a *Footer* section.

CERTIFICATE OF ANALYSIS TEMPLATE

Listed below is a template for the content and format of a COA.

Header

- Titled "Certificate of Analysis"
- Company Name, Address, Phone Number, and Identity of Manufacturer and Manufacturing Site
- Name (compendial/trade) of Excipient
- Grade of Excipient
- Compendial Designation

Body

- Lot/Batch Number
- Date of Manufacture
- Product Code or Number
- Expiration Date (if required)
- Recommended Re-Evaluation Date (if required)
- Stability Statement (if required)
- Customer Required Information

Analysis

- Test Name
- Test Results
- Acceptance Criteria (i.e., specifications)
- Reference to the Test Method
- Reference to Skip-Lot Testing (if appropriate)
- Reference to Average or In-Process Test Results (if appropriate)
- Date Retested (if appropriate)
- Summary of Noncompendial Testing (if any)

Certification and Compliance Statements

- GMP Compliance
- Additional Regulatory References

General Chapters

- Potential to Meet Additional Compendial Standards
- Content Listing and Grade of Ingredients (if a mixture)
- Other Specific Compliance Statements [e.g., organic volatile impurities (OVI), residual solvents, transmissible spongiform encephalopathy (TSE), etc.]

Footer

- Identity of Authorized Individual for Approval
- Date of Approval
- Page Number (i.e., 1 of __)

COMPENDIAL DESIGNATION

For a supplier to claim a compendial grade on the COA for an excipient, two requirements should be met. The first requirement is that the excipient be manufactured according to recognized principles of GMPs (see *General Notices and Requirements*). Adequate conformance to GMPs should also be demonstrated for subsequent steps in the distribution of the excipient. The second requirement is that the excipient meet all the specifications contained in the appropriate compendial monograph, unless its difference is stated on its label, as defined under *General Notices and Requirements*. When an excipient is listed as compendial grade, it is understood that the above requirements have been met for the material, and the user would be able to confirm this through an appropriate audit of the supplier.

Compendial standards define what is considered an acceptable article and also give test procedures that demonstrate that the article is in compliance. These standards apply at any time in the life of the article from production to consumption. The supplier's release specifications and compliance with GMPs are developed and followed to ensure that the article, when stored according to recommended conditions, will comply with compendial standards until its expiration or recommended re-evaluation date.

Every compendial article shall be so constituted that when examined in accordance with these assay and test procedures, it meets all the requirements in the monograph defining it, as well as meeting any provisions under *General Notices and Requirements* and in the general chapters, as applicable. However, it is not to be inferred that application of every analytical procedure in the monograph to samples from every production batch is necessarily a prerequisite for ensuring compliance with compendial standards before the batch is released for distribution.

Data derived from manufacturing process validation studies and from in-process controls may provide greater assurance that a batch meets a particular monograph requirement than analytical data derived from examination of finished units drawn from the batch. On the basis of such assurances, the analytical procedures in the monograph may be omitted by the supplier when judging compliance of the batch with the compendial standards.

DATES ON A CERTIFICATE OF ANALYSIS

Part of the overall goal to standardize COA for excipients includes a provision for the consistent reporting of appropriate, meaningful, and well-defined dates. The discussion below indicates specific dates that are expected on the COA, along with definitions of the dates, in order to provide suppliers and users of excipients with a mutual understanding of their meaning. Use of the recommended terminology will be helpful in reducing the number of questions on dating information reported for excipients. Use of terminology other than that discussed below is discouraged, because the terms may be ill-defined and have different meanings for the excipient supplier and user. Examples of such terms that should **not** be used include “shelf life”, “use-by date”, “warranty date”, and “expiration period”.

In reporting dates on COA for excipients, it is important that a clear and unambiguous format be used to prevent possible misinterpretation. To accomplish this, it is recommended that an alpha designation be used for the month (may be abbreviated), rather than a numerical representation. It is also recommended that the year include all 4 digits (e.g., Jan. 1, 2005, or 1 Jan. 2005).

Date of Manufacture—The date of manufacture should be included on the COA for each excipient lot and should be assigned by the suppliers on the basis of their established policies and procedures. It is recognized that excipients may be manufactured using a variety of processes (e.g., continuous or batch) that may require a period of several days or more to complete. In addition, some excipients may be mixtures or blends of other excipients, and excipient production may include reprocessing steps. Because of this diversity, the date of manufacture should be clearly defined by the supplier and consistently applied for the particular excipient and process. In reporting the date of manufacture, the excipient supplier should indicate the date of completion of the final manufacturing process (as defined by the supplier).

It is important to note that repackaging alone is not considered a processing step to be used in determining the date of manufacture. To provide traceability for a specific excipient lot, other dates may be required in addition to the date of manufacture in order to reflect additional steps such as repackaging.

Expiration Date and Recommended Re-Evaluation Date—The stability of excipients may be an important factor in the stability of the finished pharmaceutical dosage forms that contain them. Many excipients are very stable and may not require extensive testing to demonstrate continued conformance to appropriate specifications. Other excipients may undergo chemical, physical, and microbiological changes over time that cause the material to fall outside established specifications.

Appropriate expiration and/or recommended re-evaluation dates for excipients should be established from the results of a documented stability-testing program or from historical data. The testing program should include defined and controlled storage conditions (e.g., temperature and humidity), a consideration of different packaging types that may be used as market containers, and meaningful, specific test methods to adequately assess the stability characteristics of the excipient. Stability testing should determine whether possible degradation, moisture gain or loss, viscosity changes, or other possible changes occur to make the excipient unacceptable for use (e.g., unstable or hygroscopic materials). For additional information on excipient stability, see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩.

The expiration date for an excipient is defined as the date after which the supplier recommends that the material should not be used. Prior to the assigned expiration date, the excipient is expected to remain within established specifications, if stored according to the supplier's recommended conditions.

The recommended re-evaluation date for an excipient is the date suggested by the supplier after which the material should be re-evaluated to ensure continued compliance with specifications. Re-evaluation of the excipient may include physical inspection and appropriate chemical, physical, and microbiological testing. Prior to the re-evaluation date, the excipient is expected to remain within established specifications, provided it has been stored according to the supplier's recommended conditions. But beyond the recommended re-evaluation date, the excipient should not be used without adequate evaluation at appropriate intervals, to determine whether the material continues to be acceptable for use. The recommended re-evaluation date differs from the expiration date in that the excipient may be re-evaluated to extend the length of time the material may be used, if supported by the results of the evaluation and appropriate stability data.

In reporting the expiration and recommended re-evaluation dates, the excipient supplier is providing important information to the user about the stability of the material. As discussed previously, the assignment of an expiration date and a recommended re-evaluation date should be based on appropriate evaluation of potential changes that may occur in the material's properties. It is acceptable to report both an expiration date and a recommended re-evaluation date on the COA for excipients, if applicable, but both dates may not always be required. Expiration and recommended re-evaluation dates should not be reported by a supplier without sufficient stability data or product history to support the assigned dates.

For excipients determined to be very stable (greater than 2 years), either the specific expiration date and/or the recommended re-evaluation date should be reported on the COA for the material, or a general stability statement may be included (e.g., stability greater than 2 years). If available data indicate that an excipient has limited stability (2 years or less) under anticipated storage conditions, a specific expiration date and/or recommended re-evaluation date should be reported on the COA for the material.

If data from formalized stability studies are not available for an excipient, an appropriate statement should be included on the COA to indicate what is known about the stability of the material and whether stability studies are in progress.

Date Retested—If retesting is performed by an excipient supplier and the results are used to extend the length of time that the material may be used, the date retested should also be reported on the COA. The specific tests that were subject to retesting should be clearly identified, and the results obtained upon retesting should be reported. After retesting, a new recommended re-evaluation date should be reported on the COA.

Additional Dates—Other dates may appear on a COA, if desired by the excipient supplier or requested by the user. Examples include the release date, shipping date, date of testing, and date the COA was printed or approved. Any additional dates that appear on a COA for excipients should include a clear indication of what the date represents or means.

TESTING FREQUENCY

For the excipients listed in the *USP–NF*, the product specifications are set by the supplier to include all parameters listed in the monograph. It is not required that analysis of all specification parameters be made on each lot (see *General Notices and Requirements*). However, sufficient analysis and process validation data should exist to ensure that the lot meets all specifications before it is released. This is an established practice that has been successfully used in industry for many years. Periodic testing of all parameters should be performed to revalidate the control system. The frequency of these periodic tests should be determined by the suppliers on the basis of their understanding of the manufacturing control system. At a minimum, the parameters should be checked once a year.

For excipients that are not included in *USP–NF*, specifications should be set by the supplier to ensure that the quality of the material is maintained on a continuing basis and reflects both the excipient manufacturing process and inherent properties. The analytical methods used to evaluate the characteristics of noncompendial excipients may be the same as those contained in the compendia, or may be unique to the supplier or the material. The methods should be demonstrated to provide accurate, reproducible, and consistent results for the characteristic being tested. It may be appropriate for noncompendial excipients to have some tests performed at reduced frequency.

The excipient user should evaluate the supplier's specifications and methods to ensure that they are appropriate and acceptable for the quality control needed for the manufacturing process of their drug product. The user should determine which of the supplier's specifications and methods are required for release of the excipient for use in their process. If additional tests or alternative methods are required by the user, appropriate specifications and methods, along with responsibility for performing the testing, must be agreed upon by the excipient supplier and user.

Reduced Frequency Testing

When analysis of some parameters is carried out at a reduced frequency (for example, every 10th lot), this should be clearly stated on the COA. Each specific test subject to reduced frequency testing should be indicated. Reduced frequency testing should be used only for excipients manufactured using a stable process. There should be a sound technical basis and sufficient documentation to support testing any parameter at a reduced frequency. This would normally include the following points:

- Appropriate validation of the manufacturing process
- Process control—attribute charting (when appropriate)
- GMP controls

As part of the justification for reduced testing, it is important that there be assurances in place showing that the manufacturer's process complies with appropriate excipient GMP requirements.

Some tests, because of their significance, should always be performed on each lot, whereas others may be candidates for reduced frequency testing. Attribute testing results in qualitative data that provide pass/fail results or results expressed as less than or greater than a specified value. The result merely establishes compliance with a specification parameter. There are no data to indicate how well the material complies, as would be obtained from variable or quantitative test results.

Reduced frequency testing of an attribute requires that the manufacturer show that the qualitative parameter is in a state of statistical control. This necessitates tabulating the test results for consecutive lots produced.

Skip-Lot Testing—Skip-lot testing may be applied to an excipient that is made by either a batch or a continuous process. Various commonly accepted statistical sampling plans may be used to demonstrate appropriate process control. Examples of each are listed below.

EXAMPLE 1: For an average outgoing quality level (AOQL) of 1% and a test frequency of 1 in 10, the supplier should find 100 consecutive lots in conformance. At a 2% AOQL and a test frequency of 1 in 10, the supplier would test 50 consecutive lots. For a 1% AOQL and a 1 in 5 test frequency, the supplier would test 70 consecutive lots. Nomographs are available to determine the test requirements.

EXAMPLE 2: When the excipient is manufactured by a continuous process, no discrete lot is produced. The sampling plan again is based upon the risk of approving a lot that was nonconforming. By testing 140 consecutive lots before going to a test frequency of 1 in 10, the plan establishes a low risk of approving a lot that is noncompliant.

Once the requirement is met, the supplier can monitor conformance to the specification parameter by testing 1 in 10 lots. Should any lot fail the analysis, the supplier should return to 100% testing until the results once again meet the specification above.

Because excipients vary greatly in chemical and physical properties, the supplier of the excipient should determine which tests should be routinely performed and which tests may be appropriate for reduced frequency testing. This determination must be justified and documented on the basis of the adequacy of the supplier's control system. Documentation should be kept detailing the assumptions and the data supporting the skip-lot testing plan.

Type A and Type B Tests—Only certain types of tests are appropriate for reduced frequency testing. *Type A* is defined as tests that may not be easily controlled through standard process control techniques or that may change with time. These tests should normally be performed on each lot. *Type B* is defined as tests that normally can be controlled using standard process control techniques and that are not expected to change with time. These tests are candidates for reduced frequency testing. Examples of both types of tests are listed below.

TYPE A: EXAMPLES OF TESTS THAT TYPICALLY NEED TO BE PERFORMED ON EVERY LOT

- *Identification*—Required by GMPs for users (candidate for reduced frequency testing by suppliers)
- *Assay*—Critical quality parameter (if specified)
- *Viscosity*—Usually indicates grade
- *Loss on drying* (or moisture determination)—Indication of stability and appropriate process controls
- *Color*—Indication of stability and appropriate process controls
- *pH*—Indication of stability and appropriate process controls

TYPE B: EXAMPLES OF TESTS THAT MAY BE CANDIDATES FOR REDUCED FREQUENCY TESTING

- *Manufacturing impurities*—Based on starting materials and processes (e.g., Chloride, Sulfate, Nitrate, Glyoxal)
- *Heavy metals*
- *Lead*
- *Arsenic*
- *Residue on ignition*
- *Residual solvents*

This is not meant to be an exhaustive list of tests. It simply provides some direction on how a supplier can assess the importance of each test to the overall control of the process. Tests listed as possible candidates for reduced frequency testing (*Type B*) may need to be routinely tested (*Type A*), depending on the raw materials and process. Determinations can also be made for some *Type A* tests to become *Type B* tests. In a dedicated facility, identification testing by the supplier may not be necessary.

Documentation—The supplier of an excipient should develop and maintain documentation that outlines the process control systems and validation data to justify the use of reduced frequency testing. This documentation should also include procedures for handling the impact of significant changes on the reduced frequency testing program.

General Chapters

The minimum number of lots to be fully tested for all specification parameters after a change has been made depends on the process and the significance of the change and should be based on sound statistical considerations.

Additionally, the documentation should contain procedures for re-evaluating the reduced frequency testing program when a testing failure occurs. Decisions regarding the continuance of reduced frequency testing should be justified on the basis of the reasons for the failure and the supplier's ability to provide assurances that the reduced frequency testing program or other in-process parameters would identify these types of failures in the future.

Justifications for Reduced Frequency Testing—The following are examples of situations where a sound technical basis can be demonstrated and where reduced frequency testing might therefore be justified. [NOTE—There may be other such examples.]

- An impurity, by-product, or unreacted raw material could not be present in the product because the raw materials and chemical reactions used could not contain or generate such substances above the specified limits.
- The process capability index (*Cp*) on the relevant parameter is high and based on a stable process. Statistical analysis of the reduced frequency data should show that the property remains stable and within specifications. A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both Pharmacopeia-specific and customer-specific) and is thus acceptable for its intended use. For continuous processing, it is also important to demonstrate that the material has been produced under conditions in which the process has achieved a form of "steady state", i.e., in which there is minimal operator intervention and in which the in-process parameters have been stabilized (see *Appendix 2* for further definition of this concept and for determining levels of control).
- For a continuous process, the in-process analyses show that the property that is determined at reduced frequency is stable and within specification. Repeating the test on each lot would be redundant.
- An analysis that is determined on every lot has been shown to strongly correlate with an analysis that is run at a reduced frequency. The correlation shows that if a lot is within specification on the first analysis, it will be within specification on the second analysis.

USE OF ELECTRONIC SIGNATURES

Because of the growing dependence on computers and the need to accommodate paperless record systems, an electronic alternative to handwritten records and signatures is suggested. Excipient suppliers have added computer information systems to enhance productivity.

The primary issue with transfer of a COA without a handwritten signature is the validation of data. There are several considerations that should be met before an electronic signature or name attachment to a COA is considered acceptable.

- Computer systems access must be limited to authorized individuals: access is gained only after inputting a user name and a password. The system should require frequent changes of each individual password.
- A confirmation of the integrity and accuracy of the information stored in the system should be completed.
- The operation of the system must be checked routinely to ensure that the correct information is transferred from the database to the printed record.
- Data entered into a database from which information is extracted for a COA should be accompanied by time- and date-stamped audit trails.

When these criteria are met, the issuance of COAs with electronic signatures or the responsible person's name attached to the document, in lieu of a handwritten signature, is acceptable. [NOTE—Computer systems are currently regulated by 21 CFR 11 of the FDA. Users should monitor the FDA's approach to compliance in this area.]

DISTRIBUTOR INFORMATION

The presentation of a COA issued by a distributor presents some challenges. Because COAs are important documents characterizing the excipients and the state of their quality, the source of that information becomes very important to the end user(s). Because distributors take on different roles in fulfilling the services for which they are contracted, it is necessary to ensure that procedures and methods are appropriate for the functions performed.

Distributors may function in a number of different capacities relating to the movement of excipients and to services associated with their production. Some are simply pass-through locations in which nothing is done to the excipient with the exception of storage and handling. Others serve as extensions of the manufacturer's process by taking bulk quantities and repackaging them for the manufacturer. Still others purchase excipients and repackage them under a different label for sale and distribution. These scenarios should be understood and properly documented with programs that will protect the integrity and safety of the excipients as they move through the distribution process.

Original Manufacturer and Manufacturing Site—The identity of the original manufacturer and the manufacturing site should be included on the COA for excipients. This information is important because it provides traceability for specific excipient lots and assures the excipient users that they are consistently obtaining material from the same manufacturer and site.

Reporting the identity and location of the manufacturer does not represent an issue when the original manufacturer is also the direct supplier of the excipient to the pharmaceutical customers. However, it is recognized that this information may be considered proprietary by an excipient distributor. To adequately address this issue, excipient distributors should either list the

specific information identifying the original manufacturer and location or provide the information by reporting an appropriate code, which is assigned in order to unambiguously identify the original manufacturer and manufacturing site. To protect the secrecy of this information, the meaning of the code does not have to be revealed to intermediary distributors.

Certificate of Analysis Data—When a distributor is primarily used as a pass-through of the excipient without any changes to the excipient and packaging, the COA that accompanies the excipient from the manufacturer can be passed on in the original form. If the data are extracted, translated, or rewritten on other letterhead, a system should be in place to check the rewritten information, and justification should be demonstrated upon request. Alternatively, the source of the data should be indicated on the document.

For a distributor that takes bulk quantities of an excipient from a manufacturer and introduces the bulk quantities into a process (e.g., conveyance and storage system), analysis of the packaged excipient should be performed to demonstrate the same quality as the lot (batch) introduced. Appropriate analytical data should be included on the COA to verify the quality. The distributor should use equivalent methodology and equipment for the analytical evaluation. Some data may be used from the original manufacturer's COA with appropriate justification.

In all scenarios, it is expected that the distributor will have the appropriate level of GMP in place.

APPENDIX 1

DEFINITIONS

Acceptance Criteria—The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.

Batch (or Lot)—A defined quantity of excipient processed so that it could be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production, based on time or quantity (e.g., vessel's volume, 1 day's production, etc.).

Batch Number (or Lot Number)—A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.

Batch Process—A manufacturing process that produces the excipient from a discrete supply of raw materials that is present before the completion of the reaction.

Certificate of Analysis (COA)—A document relating specifically to the results of testing a representative sample drawn from the batch of material to be delivered.

Chemical Property—A quality parameter that is measured by chemical or physicochemical test methods.

Continuous Process—A manufacturing process that continually produces the excipient from a continuous supply of raw material.

Contract Facility—An internal or external facility that provides services to the manufacturer or distributor of an excipient. These can include, but are not limited to, the following: manufacturing facilities, laboratories, repackaging facilities (including labeling), and warehouses.

Date of Manufacture—A date indicating the completion of the final manufacturing process (as defined by the supplier for the particular excipient and process).

Date Retested—The date when retesting is performed by an excipient supplier to extend the length of time that the material may be used.

Distributor—A party other than the manufacturer who sells the excipient.

Excipient—Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

Expiration Date—The date after which the supplier recommends that the material should not be used.

Impurity—Any component of an excipient that is not the intended chemical entity but is present as a consequence of either the raw materials used or the manufacturing process.

Lot—See *Batch*.

Lot Number—See *Batch Number*.

Manufacturer—A party who performs the final processing step.

Packaging—The container and its components that hold the excipient for storage and transport to the customer.

Periodic Testing Program—See *Skip-Lot Testing Program*.

Physical Property—A quality parameter that can be measured solely with mechanical equipment.

Process—The set of operating instructions describing how the excipient is to be synthesized, isolated, purified, etc.

Process Capability Index (*Cp*)—A statistical measurement that can be used to assess whether the process is adequate to meet specifications. A state of statistical control can be said to exist if the random variation in test results for a process parameter is such that the calculated process capability is greater than 1.33 (see *Appendix 2* for further definition).

Process Step—An instruction to the excipient manufacturing personnel directing that an operation be performed.

Recommended Re-Evaluation Date—The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. Differs from the *Expiration Date* in that the excipient may be re-evaluated to extend the length of time the material may be used, if supported by the results of the evaluation and appropriate stability data.

Reduced Frequency Testing Program—See *Skip-Lot Testing.*

Repackaging—Transfer of an excipient from one container to another.

Reprocessing—Introducing previously processed material that did not conform to standards or specifications back into the process and repeating steps that are already part of the normal manufacturing process.

Significant Change—Any change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

Site—A location where the excipient is manufactured. This may be within the facility but in a different operational area, or at a remote facility, including a contract manufacturer.

Skip-Lot Testing Program—Periodic or intermittent testing performed for a particular test parameter that is justified by historical data demonstrating a state of statistical process control.

Specification—The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

Stable Process—A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP-specific and customer-specific) and is thus acceptable for its intended use.

Supplier—A manufacturer or distributor who directly provides the excipient to the user.

User—A party who uses an excipient in the manufacture of a drug product or another excipient.

APPENDIX 2

STATE OF STATISTICAL CONTROL: PROCESS CAPABILITY PARAMETERS FOR DETERMINING LEVELS OF CONTROL

A process is considered to be in a state of statistical control if variations among the observed sampling results from the process can be attributed to a constant system of chance causes. Process capability index (Cp) or capability index adjusted for the process average (Cpk) or performance index (Pp) or performance index adjusted for the process average (Ppk) can be used to assess whether the process is adequate to meet specifications. Values of these parameters exceeding 1.33 show that the process is adequate to meet specifications. Values between 1.00 and 1.33 indicate that the process, although adequate to meet specifications, will require close control. Values below 1.00 indicate that the process is not adequate to meet specifications and that the process and/or specifications should be changed. Pp/Ppk will always be less than or equal to Cp/Cpk, respectively. The essential difference between the capability and the performance indices is the data used. Capability indices require the calculation of σ, the population standard deviation, whereas the performance indices require the calculation of s, the sample standard deviation. Thus for pharmaceutical excipients a state of statistical control can be said to exist if the random variation in test results for a process parameter is such that the calculated process capability index or performance index is greater than 1.33.

⟨1084⟩ GLYCOPROTEIN AND GLYCAN ANALYSIS—GENERAL CONSIDERATIONS

OVERVIEW

A number of glycoprotein drugs have been developed as a result of advances in biotechnology, and many naturally derived protein drugs possess complex glycan structures. Glycosylation, a posttranslational modification of these proteins, can play an important role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of these agents. The two main types of protein glycosylation are *N*-glycosylation and *O*-glycosylation. Unlike transcription and translation, glycosylation is not a template-driven process; therefore variability in the glycosylation pattern of a protein can arise, caused by different sources or different manufacturing processes. Differences in this pattern are known to affect biological activity. Glycosylation patterns may therefore be an important set of attributes that arise in characterizing a candidate glycoprotein intended for therapeutic use and in ensuring its stability and quality.

The first part of this chapter provides a brief introduction to glycobiology and describes the complexity of glycan structures. The subsequent parts provide flow charts and a series of general analytical strategies that can be used to characterize glycoprotein glycans by means of the following:

1. Direct analysis of glycoproteins; and

2. Analysis of released nonderivatized or derivatized glycans by various methods of chromatographic and electrophoretic separation and mass spectrometry (MS).

Different approaches to analyzing monosaccharides are described at the end of the chapter.

For selected analytical methods, this chapter cross-references other USP chapters, particularly those relating to biotechnology-derived articles (see chapters *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩).

PROTEIN GLYCOSYLATION

Most proteins in eukaryotic cells undergo glycosylation and other posttranslational modifications before being trafficked to lysosomes, becoming membrane bound at the cell surface, or being secreted. Glycosylation varies significantly from cell to cell, tissue to tissue, and species to species because of the varying expression of hundreds of glycosyltransferases and glycosidases located throughout the Golgi apparatus and endoplasmic reticulum (ER). Four main types of enzymatic glycosylation are found in proteins:

1. *N*-Glycosylation, which involves the initial transfer of oligosaccharides to the nitrogen on the terminal amide group of asparagine and their subsequent processing and modification to a series of glycan chains;
2. *O*-Glycosylation, which in general involves the initial transfer of monosaccharides to the hydroxyl groups of serine and threonine and subsequent elongation and branching of the saccharide chain by the addition of monosaccharides;
3. Glycosylphosphatidylinositol (GPI) anchor, which is a glycolipid linked to the C-terminus of a protein; and
4. C-Glycosylation, which involves the formation of a carbon–carbon bond between the C2 carbon of the indole ring of tryptophan and the C1 carbon of an α-mannopyranosyl residue.

Any given protein may contain multiple *N*-, *O*-, or *C*-glycosylations, but not more than one GPI anchor. A nonenzymatic addition of saccharides, called glycation, can occur when proteins are mixed with reducing sugars via a complex series of reactions. The two protein glycosylation types that are generally of concern and that are analyzed in glycoprotein drug substances are *N*- and *O*-glycosylation. Each of these is discussed below.

N-Glycosylation

The biosynthesis of *N*-glycans in glycoproteins can be described as a four-step process:

1. Lipid-linked glycan chain initiation and elongation;
2. Transfer of oligosaccharide to the protein or nascent polypeptide chain;
3. Processing of the *N*-glycan chain by removal of specific glucose and mannose residues; and
4. Modification of the *N*-glycan chain by the addition of residues to the nonreducing ends of the glycan chain.

The consensus amino acid sequence for *N*-glycosylation is Asn–Xaa–Thr/Ser (where Xaa is any amino acid other than proline). Overall, only about two-thirds of all potential sequences, termed sequons, are glycosylated, and currently there is no method to predict which sequon will be glycosylated. The role of protein *N*-glycosylation is usually protein trafficking and secretion.

N-glycans can be categorized as high-mannose, hybrid, or complex, depending on the extent of processing *(Figure 1)*. High-mannose structures (*Appendix 1*) lack galactose or *N*-acetylglucosamine (GlcNAc) residues in the antennae, branches at the distal end of the chain. In hybrid structures, both substituted GlcNAc residues and terminal mannose residues are present in the antennae, whereas complex structures have both α1,6- and α1,3-mannose residues substituted with GlcNAc moieties. Hybrid and complex glycans can exist with two or more branches, frequently termed antennae; such glycans are therefore often termed, for example, biantennary, triantennary, or tetraantennary. Both monoantennary and pentaantennary *N*-glycans are also known to exist. In complex glycans, antennae frequently carry terminal sialic acid (neuraminic acid) residues. Sialylation has been shown to have a great effect on both the pharmacokinetics and the pharmacodynamics of many therapeutic glycoproteins.

Common type of asparagine (Asn) linked glycans (*N*-glycans).

Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid

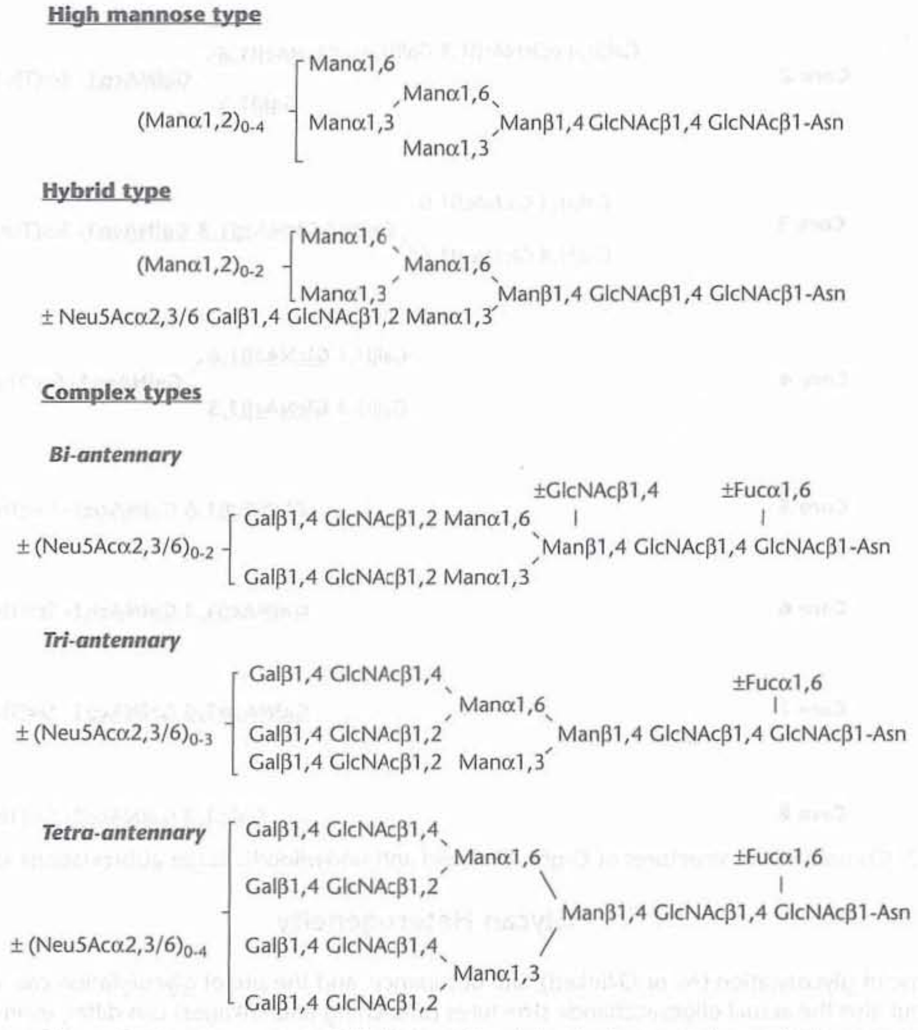


Figure 1. Common types of *N*-glycans. For abbreviations, see *Appendix 1*.

O-Glycosylation

O-Glycan chains are built up sequentially via an initial GalNAc residue linked to serine, threonine, and tyrosine, as well as to the less common amino acids hydroxyproline and hydroxylysine. Multiple glycan core structures are known. The sequence and isomeric linkage of monosaccharides show greater variety than that in *N*-glycans, and at least eight different types have been identified *(Figure 2)*. Although no consensus amino acid sequence for *O*-glycosylation has been determined, glycosylation is usually favored by the presence of proline one residue before or three residues after the glycosylation site and the absence of charged amino acid residues proximal to serine or threonine. The disaccharide unit *N*-acetyllactosamine, Galβ 1,4GlcNAc, is the most common chain extension. Additional modifications, including terminal capping of Gal with sialic acid and fucosylation along the chain, are also frequent.

O-Glycosylation can occur in cluster form, the mucin type, which usually forms part of the cell surface extracellular matrix or secreted glycoproteins. Other *O*-glycosylation, such as *O*-GlcNAc, is found on many nucleocytoplasmic proteins; and *O*-Man–linked glycosylation is found in some muscular and neural glycoproteins and in yeast. *O*-Fuc– and *O*-Glc–linked glycosylation types are found on many epidermal growth factor-like proteins that are associated with the Notch signaling pathway.

Figure 2. Common core structures of *O*-glycans (bold and underlined). Sugar abbreviations as in *Appendix 1*.

Glycan Heterogeneity

Not only the type of glycosylation (*N*- or *O*-linked), site occupancy, and the site of glycosylation can vary from glycoprotein to glycoprotein, but also the actual oligosaccharide structures (branching and linkages) can differ, even on the same site. This structural variation arises because glycosylation is a process that is not driven by a template. The glycosylation pattern at a given site depends on many factors, including cell-specific and growth-dependent availability of glycosyltransferases and *exo*-glycosidases found in the Golgi bodies and ER. Heterogeneity leads to different physical and biochemical properties and, therefore, also to functional diversity.

Host-Cell Expression Systems and Glycosylation

BACTERIA

Although both *O*- and *N*-glycosylation have been shown to occur in a variety of prokaryotes, *Escherichia coli*, the bacterium of choice for many therapeutic products, does not produce glycosylated proteins.

YEAST

Yeast produces both *N*-glycosylated and *O*-glycosylated proteins. In yeast hypermannosylation with the *N*-glycan chain that contains more than 100 mannose residues can occur, but sialylation does not occur unless the organism is genetically modified. The development of recombinant strains of *Pichia pastoris* that contain inserted heterologous genes for various glycosylation enzymes has allowed the humanization of *N*-glycosylation pathways in this yeast. *O*-Glycosylation in yeast is also significantly different from that in mammalian cells. In contrast to mammalian cells, serine or threonine *O*-glycosylation is linked via mannose and often consists of linear chains of as many as six mannose residues.

INSECT CELLS

N-glycan chains of insect cells usually are of the high-mannose, trimannose or paucimannose, and truncated complex types (see *Appendix 1* for definitions). Insect cells also produce glycoproteins bearing the Fucα1,3 residue linked to the proximal GlcNAc residue in the core chitobiose. This core fucose residue is a potent immunogen and allergen. *O*-Glycosylation in insect cells has not been well-studied, and although *O*-linked GalNAc–Ser(Thr) residues have been found, very few are processed further beyond the Galβ1,3GalNAc–Ser(Thr) sequence. Sialic acid residues have not been found on proteins produced in insect cells.

PLANTS AND PLANT CELLS

Plant *N*-glycans contain mainly oligosaccharides of the oligomannose type, but also present are hybrid and truncated complex types of structures, with or without Xylβ1,2 attached to the β-linked mannose residue of the trimannosyl core and Fucα1,3 attached to the proximal GlcNAc residue of the core chitobiose. Both the Fuc and Xyl residues are immunogenic and have been shown to be part of the glyco-epitopes of several plant allergens. *O*-Glycosylation in plants has not been well studied but is known to consist predominantly of the addition of arabinogalactan chains attached to hydroxyproline, threonine, and serine residues that are located in the plant cell wall or on the outer surface of the plasma cell membrane. These glycans are immunogenic.

ANIMAL CELLS

The majority of glycosylated therapeutic proteins are produced in continuous animal cell lines. Chinese hamster ovary (CHO), baby hamster kidney (BHK), human embryonic kidney (HEK), and mouse myeloma (SP2/0 or NS0) cells have all been employed. These animal cells generally produce proteins with humanlike glycosylation. Although there are several differences in glycosylation between rodent and human cells, such as the presence of *N*-glycolylneuraminic acid not found in humans, CHO cells have become a workhorse of the biotechnology industry.

GLYCAN ANALYSIS FOR GLYCOSYLATED BIOLOGICAL DRUGS

Glycosylation of proteins may affect biological activity, either directly or indirectly, and variability in glycosylation arises not only from cellular diversity but also from the manufacturing process. The glycosylation pattern thus may be important as a part of characterization studies in assuring process consistency and may be also be important in ensuring the consistent quality of a biological drug product after market access. Appropriately characterized reference materials are needed in order to support biological and physicochemical testing of production batches to ensure batch-to-batch consistency. Glycosylation analysis may be appropriate for the following:

1. Characterizing the structure and stability of novel products and their stability to processing steps and storage;
2. Batch release testing and in process control testing; and
3. Assessing comparability between products (e.g., when one or more process changes have been made).

An understanding of the relationship between glycan structure and biological function underpins decisions about the information required at each development stage. For biological/biotechnological drug substances, the characterization criteria and specifications for batch release are generally set forth in the guidelines ICH Q6B, *Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*;[1] and ICH Q5E, *Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process*.[2] Numerous approaches and methodologies are applied for glycan mapping. This variety is a consequence of the diversity and complexity of glycan structures and the available technology and detection systems.

Because of the diversity and complexity of glycan structures and the increasing availability and improvement of various detection systems and technology, analytical methods are wide ranging. Different methods that support step-by-step procedures depend on the glycoproteins, the availability of equipment, the expertise of individual scientists and groups, and the information required. The two most studied types of protein glycosylation that affect bioactivity are *N*- and *O*-glycosylation.

Glycan analysis can serve in different applications; the most important are general product characterization, process validation, comparability evaluation, stability testing, monitoring manufacturing process consistency, and release testing. The selection of the analytical techniques and their applications in product development and routine manufacturing depend on many factors, such as the complexity of the glycoprotein, the understanding of the relationships between glycosylation and safety and efficacy, and the overall design of the strategy for manufacturing process control. For example, even when the biological relevance of glycosylation is not certain, control of glycosylation could be considered as a measure of manufacturing consistency.

The *Figure 3A* flow chart assists in the choice of applications for glycan analysis, and *Figure 3B* provides an overview of available analytical techniques and equipment employed.

[1] http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html; accessed 12/15/2011.
[2] http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html; accessed 12/15/2011.

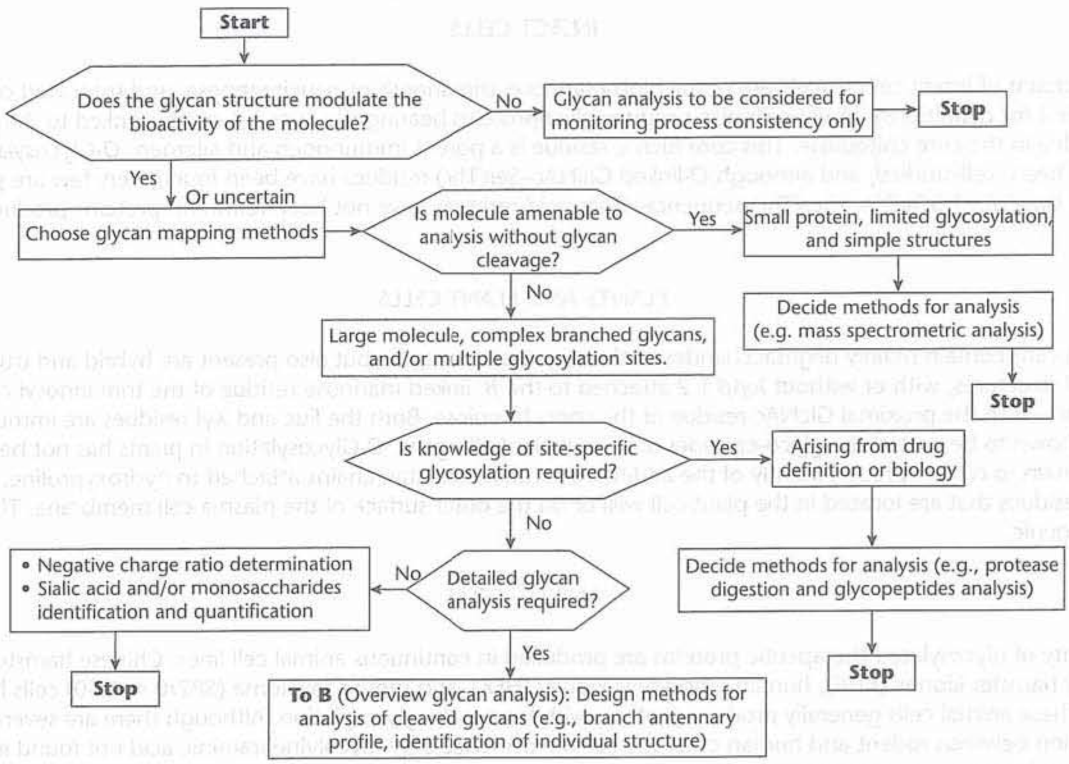


Figure 3A. Flow diagram assisting in the choice of options for glycan analysis.

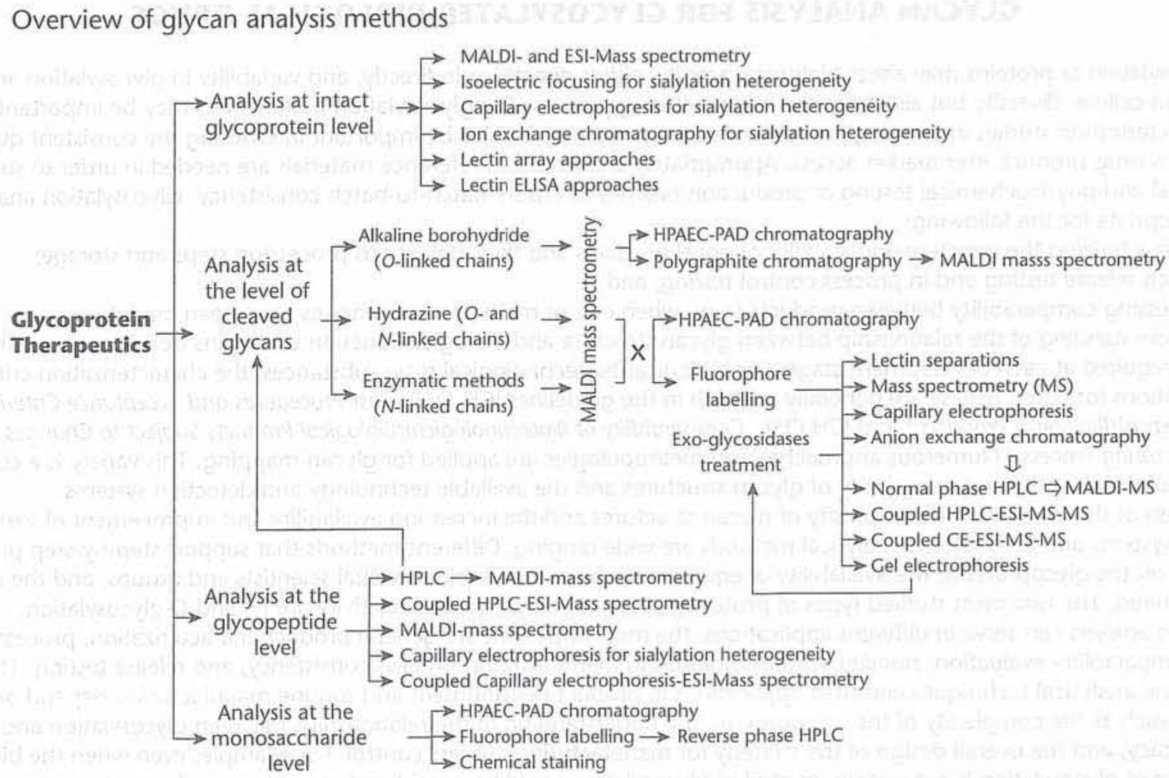


Figure 3B. Overview of glycan analysis techniques and equipment employed.

CHOICE OF GLYCAN ANALYSIS FOR CHARACTERIZATION AND SPECIFICATION OF GLYCOSYLATED BIOLOGICAL DRUGS

Analysis of Intact Glycoprotein

The most direct mode of analysis is direct study of the intact molecule. This mode provides information about the glycosylation profile of the glycoprotein. However, this approach provides limited information when the molecule is large and contains multiple glycosylation sites. One of the most important glycosylation factors defining biological activity is the degree of sialylation, which often determines the half-life of glycoproteins in circulation. This makes ionic-charge–based electrophoresis and ion-exchange chromatography obvious choices of technique. Nearly all types of gel electrophoresis have been used to probe protein glycosylation, including polyacrylamide gel electrophoresis (PAGE), (see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* 〈1056〉) and isoelectric focusing (IEF), (see *Biotechnology-Derived Articles—Isoelectric Focusing* 〈1054〉). Similarly, capillary electrophoresis (CE), (see *Capillary Electrophoresis* 〈1053〉) has also been found suitable. Strong anion-exchange chromatography has been used for the same purpose, but the resolution is often inferior to that of IEF and CE. Direct mass spectrometry (MS) is another option for the analysis of posttranslational modification. Along with ongoing improvements in the resolution of MS, more and more complex glycoproteins become accessible for direct characterization by this method.

Analysis of Glycopeptides

Analysis of glycopeptides provides information about site-specific glycosylation properties, the degree of occupancy, and oligosaccharide structures. Site-specific glycosylation can be affected by cell culture process conditions. Therefore, if a known glycosylation site is critical, manufacturers must monitor site-specific glycan structures. The typical approach is first to generate glycopeptides by protease digestion and to separate them by, for example, RP (reversed-phase) HPLC (see *Biotechnology-Derived Articles—Peptide Mapping* 〈1055〉). Subsequently the separated glycopeptides can be further characterized individually by, e.g., direct analysis using MS, or deglycosylation and subsequent glycan profiling, as described below in the section *Profiling of Cleaved Oligosaccharides*.

Direct identification of the mixture of glycopeptides and nonglycosylated peptides by MS is limited by masking effects (ion suppression) of peptide signals on glycopeptide signals. One approach to overcoming this effect is to separate peptides and glycopeptides before analysis by MS, e.g., by offline coupling (matrix-assisted laser desorption ionization [MALDI]) or online coupling (electrospray ionization [ESI]). MS analysis of glycopeptides plays an important role in the characterization of *O*-glycans because these glycans are not always released quantitatively and because, as a result of their smaller size, they are more amenable to characterization by MS as glycopeptides.

The use of CE for high-resolution separation may also be appropriate, especially for the analysis of sialylation.

Profiling of Cleaved Oligosaccharides

Profiling of total glycans cleaved from glycoprotein is the most common approach for the characterization of glycoproteins. It provides a way to obtain information about the various populations of glycans present on the protein. The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatization/labeling may be needed to allow the detection of the glycans. Many protocols are available, and most of the steps in the analysis are well established. The possible drawback with such flexibility is the lack of consensus about which methods to choose under which circumstances; because of the variety of analytical techniques, comparison of results obtained by different platforms may not always be possible. So far, the majority of the work has been done on *N*-glycosylation, because of the following factors:

1. *N*-glycans usually are more clinically relevant in biologicals than *O*-glycans; or
2. The release of *N*-glycans, either by chemical means (hydrazine) or by enzymes (endoglycosidases and peptide *N*-glycosidase [PNGase] F), is more straightforward than is the release of *O*-glycans.

DEGLYCOSYLATION

The approach used for the release of glycans depends on the glycoprotein under test. The cleavage agent is chosen according to the type of cleavage needed and the level of information required. Enzymatic or chemical cleavage may be used. *Table 1* gives a nonexhaustive list of enzymatic cleavage agents and their specificity. Digestion efficiency generally depends on the accessibility of the glycans on the protein, and hence the protein should be denatured to maximize glycosylation site exposure unless analysts want to distinguish between surface and buried glycans. Chemical cleavage agents can also be used, e.g., hydrazine or alkaline borohydride for β-elimination of *O*-linked glycans.

General Chapters

Table 1. Examples of Enzymatic Cleavage Agents

Agent	Specificity
N-linked glycan release	
Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52)	Hydrolysis of peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) *N*-acetyl-β-D-glucosaminylamine and a peptide containing an aspartate residue
Peptide *N*-glycosidase F (PNGase F)	Release of *N*-glycan chain but no release of *N*-glycan chain containing (α1,3)-linked core fucose
Peptide *N*-glycosidase A (PNGase A)	Release of *N*-glycan chain containing (α1,3)-linked core fucose
Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96)	Endohydrolysis of the *N,N'*-diacetylchitobiosyl unit in high-mannose glycopeptides/glycoproteins containing the –[Man(GlcNAc)$_2$]Asn structure
Endo-β-*N*-acetylglucosaminidase F (endo F)	Release of high-mannose, hybrid, and complex oligosaccharides
Endo-β-*N*-acetylglucosaminidase H (endo H)	Release of high-mannose and hybrid oligosaccharides
O-linked glycan release	
Glycopeptide α-*N*-acetylgalactosaminidase (EC 3.2.1.97)*	Hydrolysis of terminal D-galactosyl-*N*-acetyl-α-D-galactosaminidic residues

* This enzyme has limited usage because of its high substrate specificity.

Chemical or Enzymatic Release of *N*-Glycans—PNGase F (*Flavobacterium meningosepticum*) is the enzyme of choice for the release of *N*-glycans for most glycoproteins except for some insect cell and plant glycoproteins that may contain a Fucα1,3 linked to the chitobiosyl core. *N*-Glycan chains having this structure can be cleaved from the glycopeptide only by the almond enzyme, PNGase A. Chemical release by anhydrous hydrazine is much less common, mainly because of the limited availability of the reagent, which is considered a hazardous chemical. In addition, hydrazinolysis produces de–*N*-acetylated *N*-glycans.

Chemical or Enzymatic Release of *O*-Glycans—Currently only one enzyme, *O*-glycanase from *Diplococcus pneumoniae*, is available to release *O*-glycans, and this enzyme has a limited usage because of its high substrate specificity: it cleaves only Galβ1,3GalNAcα1-Ser/Thr. In addition, no ideal chemical procedure is available; but Ser- and Thr-linked *O*-linked glycan can usually be released by the reductive alkali-catalyzed β-elimination reaction (alkaline borohydride reaction), in which the released glycans are reduced as soon as they are cleaved in order to prevent formation of degradation products due to peeling. However, this reaction is not specific, and in the reaction, approximately 10%–20% of *N*-glycans are generally known to be released as well. The released glycans lack a reducing group used for the attachment of fluorescent labels by reductive amination. Fortunately, with advances in sensitive MS, direct identification of reduced glycans is possible. Relatively good quality reducing *O*-glycans can be obtained by alkali-catalyzed β-elimination using primary amines such as ethylamine and hydrazine. However, both reagents have the potential to produce peeled degradation products. Furthermore, *O*-glycan release by ethylamine is not quantitative. Hydrazine, although it may be better for use than ethylamine, requires strict control of reaction conditions and handling and does not have a commercial source in Europe.

Separation of Cleaved Glycans Without Fluorescent Labeling—*N*-Glycans can also be resolved by HPAEC high-pH anion-exchange chromatography with pulsed amperometric detection (PAD; see *Chromatography* ⟨621⟩), which shows high sensitivity, can also separate some isomers, and affords the ability to directly detect native glycans without labels or tags. However, LC/MS for this separation approach is challenging because this HPAEC system uses high-pH and high-salt mobile phases that interfere with ionization of glycans. In addition, absolute quantification of the glycan is only possible if the individual PAD response factors for the different glycan structures are known, e.g., if an appropriate oligosaccharide reference library is available. Porous graphitic carbon (PGC) chromatography can also be used to separate glycans, and this method adds an orthogonal selectivity compared to other columns. A PGC-electrospray-ionization-MS approach also can be applied for direct glycan analysis.

MALDI/ESI-MS is a powerful method for the analysis of glycan mixtures either in the native or derivatized form. Permethylation of released glycans is a common method for direct analysis using MALDI/ESI-MS especially for sialylated glycans.

LABELING OF GLYCANS TO INCREASE DETECTION SENSITIVITY AND/OR TO MODIFY THEIR PHYSICOCHEMICAL PROPERTIES

Chemical derivatization is the most commonly used method for labeling glycans at their reducing end by reductive amination. One fluorescent label can be attached to each mono- and oligosaccharide, which facilitates determination of molar quantities. *Table 2* illustrates the most common examples of fluorescent labels and their most common uses.

Table 2. Examples of Fluorescent Labels

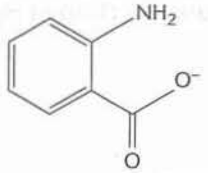
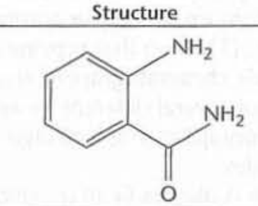
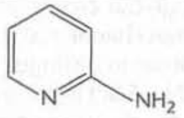
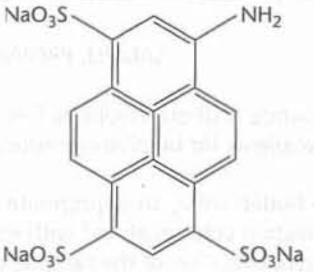
Name	Acronym	Structure	Analytical Technique
2-Aminobenzoic acid	2-AA		HPLC

Table 2. Examples of Fluorescent Labels *(Continued)*

Name	Acronym	Structure	Analytical Technique
2-Aminobenzamide	2-AB		HPLC MS
2-Aminopyridine	2-AP		HPLC
Trisodium 8-aminopyrene-1,3, 6-trisulfonic salt	APTS		CE

N-GLYCAN PROFILING

Released glycans can be analyzed or profiled by chromatographic, electrophoretic, or MS procedures and, in general, by a combination of these. The choice of method can be grouped according to the nature of the glycans and level of information required. Analysis of glycans provides information about the various populations of glycans present on the protein (high-mannose, hybrid, complex).

Profiling of Glycans by HPLC and/or by Electrophoresis and MS—Profiling of fluorescent-tag–labeled glycans by HPLC has become the most common approach. One label can be attached to every single mono- and oligosaccharide by reductive amination at their reducing end, which facilitates determination of molar quantities. With the appropriate label, glycans can be profiled with high sensitivity using reversed-phase, normal-phase, and anion-exchange HPLC (see *Chromatography* ⟨621⟩). Routinely, analysts use a combination of these methods in order to increase separation resolution and to better differentiate glycan structures. The accuracy of the glycan identification can be validated by means of glycan standards and/or by coupling the HPLC system with MS. Thus, anion-exchange, normal-phase, and reversed-phase HPLC–ESI–MS–MS form powerful combinations; and in-line analysis, if possible, may provide both relative quantitative profiling and information on glycan structure in a single run. Peak identification through retention time is acceptable if their identities have been previously validated by complementary methods and peak homogeneity can be assured.

The degree of sialylation of glycan chains can be a crucial factor for clinical efficacy, because sialylation often defines the half-life of the molecule in vivo. Anion-exchange HPLC is the simplest method for its determination, and glycan structures based on charge can then be identified by MS. Desalting of each fraction is required before MS if the ionization interface is designed for low-salt-containing sample flows only.

High-resolution separation systems such as CE have been used to identify glycan structures without MS when well characterized standards are used for comparison. The development of an online CE–MS system has further increased the power of glycan analysis using this approach.

Structural Identification by Micro-Enzyme Sequencing and Mass Spectrometry—Traditionally, when detailed structural information is required, the analysis is usually performed using micro-enzyme sequencing. This procedure is highly dependent on the specificity and quality of the enzymes used. Recently, tandem MS has been used more regularly to confirm, determine, and sequence known and novel glycan structures; this method is feasible especially when glycans are released from well-known glycoproteins and production sources.

Monosaccharide Analysis

Different quantitative monosaccharide assays are carried out for a number of purposes. In the glycoprotein field, they provide information about the relative amounts of saccharide in a glycoprotein and about the degree of sialylation of a glycoprotein; and by the measurement of monosaccharide composition, they provide some information about the structure of the glycan chains present.

The simplest assays used are colorimetric tests to demonstrate that the product is glycosylated and to quantify the total amount of saccharide present in the product. These have poor specificity between different types of sugar residues.

Assays of monosaccharide composition are generally simpler to perform than is oligosaccharide profiling, but they provide less information. The most widely used assay is quantification of sialic acid content, because loss of sialylation and exposure of terminal Gal residues may lead to faster clearance of the glycoprotein from the circulation.

The assays can be divided into two types: (1) those that provide compositional information about the intact sample without prior degradation; and (2) others, principally chromatographic, that require hydrolysis of the saccharide chains before analysis and generate quantitative information about several different monosaccharide species simultaneously. In general, the former are colorimetric and the latter are chromatographic. The hydrolysis step is a significant source of assay variability and may require careful optimization for specific samples.

The presence of certain monosaccharides is diagnostic of specific glycan structures. For example, observation of GalNAc is usually a marker for the presence of *O*-linked glycan chains, and fucose denotes the presence of specific types of chains. As a consequence of the limited diversity of monosaccharide residues present in glycoprotein glycans, accurate quantification of Man, Gal, or GlcNAc residues is required in order to distinguish between large numbers of structurally diverse glycans. The monosaccharide *N*-glycolylneuraminic acid (Neu5Gc) is not produced in humans and is generally regarded as an unwelcome and potentially immunogenic component of biopharmaceutical products.

SAMPLE PREPARATION

Glycoprotein samples for monosaccharide analysis should be free of salts, excipients, and other carrier materials (low molecular weight sugars are often used as excipients for biopharmaceuticals). This can be achieved by a number of methods, including the following:

1. Dialysis against water or a volatile buffer, using an appropriate membrane, and lyophilization;
2. HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index, and followed by lyophilization of the sample; or
3. Sample trapping on a conventional RP-SPE cartridge such as a C18 or C8 SPE system, followed by washing away of salts and excipients and elution of the required sample.

QUANTIFICATION

The common method for quantification of neutral sugars in glycoproteins depends on the color generated by heating glycans or glycoproteins in the presence of aqueous phenol in concentrated sulfuric acid. In many cases, the heat required for this reaction is generated by addition of concentrated sulfuric acid to the glycoprotein–phenol mixture in water. Rapid and efficient mixing of the solutions is critical for consistent results. Quantitative results are obtained by the simultaneous analysis of standards to generate a standard curve of absorbance against amount of saccharide and/or against a reference sample of the product under analysis.

HYDROLYSIS PROCEDURES FOR POLYSACCHARIDES AND GLYCOPROTEIN GLYCAN CHAINS

Chromatographic methods for the identification and quantification of monosaccharide components require hydrolysis of the sample before analysis. Appropriate sample preparation is required because excipients or process-related impurities may be saccharides, and residual salts may interfere with the hydrolysis or the subsequent chromatographic separation or with fluorophore labeling. Sialic acid residues can be released either by mild acid hydrolysis or by enzymatic treatment, which leaves other sugar residues attached to the peptide backbone. Quantification of the amount of saccharide present is based on addition of an internal standard before or after hydrolysis. The most commonly used standard for sialic acid analysis by HPAEC is 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), and 2-deoxyglucose is widely used for neutral sugars. Both of these sugars are acid labile and should be added after the hydrolysis step. Accurate quantification depends both on stoichiometric hydrolysis and a lack of degradation of the monosaccharide products during hydrolysis.

DETERMINATION OF TOTAL SIALIC ACIDS

Sialic acids occur in bacterial polysaccharides and glycoproteins generally as *N*-acetyl and *N*-glycolyl derivatives of neuraminic acid (Neu5Ac and Neu5Gc). The sialic acids can be determined together with other monosaccharides by a procedure that includes acid hydrolysis to liberate constituent monosaccharides, followed by HPLC using an appropriate standard mixture. Alternatively, total sialic acid content can be determined by colorimetric procedures without the need for hydrolysis. One method, commonly referred to as the Warren method, is based on the reaction of thiobarbituric acid with the product of periodate oxidation of neuraminic acid released in situ from the glycoprotein. Alternatively, the color can be generated by the reaction of resorcinol with neuraminic acid. For accurate quantification, include a reference standard sample is included in each measurement.

Selective Release of Sialic Acids—Mild acid hydrolysis or enzymatic digestion can be used to selectively release sialic acid from glycoprotein glycan chains for quantification by chromatographic methods and for quantification of unwelcome forms such as Neu5Gc. More aggressive acid conditions are required in order to release neutral and amino sugars before chromato-

graphic analysis. The protocol must be optimized with respect to yield and saccharide degradation for each protein to be analyzed.

Neuraminidase Digestion for the Release of Sialic Acid from Intact Glycoproteins—Several types of neuraminidases have been isolated and studied; the enzyme derived from *Clostridium perfringens* is the one most commonly used for the enzymatic release of sialic acids from glycoproteins. Recombinant enzyme is available from commercial suppliers. Other enzymes with different specificities are available and can be used to distinguish different types of linkages. Hydrolysis conditions should be optimized for each product, because kinetic parameters for different linkages and for Neu5Ac and Neu5Gc may differ. Selective removal of Neu5Acα2→,3-linked and Neu5Acα2→,6- from cleaved glycans is a convenient means of defining linkages. For quantitative analyses, a known quantity of a suitable internal standard, often 2-deoxyglucose, is added after hydrolysis and removal of the acid.

SEPARATION AND QUANTITATION OF UNLABELED MONOSACCHARIDES

Essentially the only method used for the simultaneous identification and quantification of unlabeled monosaccharide in hydrolysates is HPAEC-PAD. HPAEC-PAD methods are also applicable to oligosaccharide separations, and a single instrumental approach can be used for both applications.

For the general principles and components of chromatography, see *Chromatography* 〈621〉. HPAEC-PAD facilitates analysis of monosaccharides and all classes of oligosaccharides without derivatization. Carbohydrates, because they are polyhydric compounds, are weak acids that have pKa values of 12–14, and at high pH even neutral carbohydrates are ionized and can be separated as oxyanions by ion-exchange chromatography. Although separations can be performed on alkali-stable porous polystyrene–divinylbenzene anion exchangers, carbohydrates tend to exhibit broad peaks as a result of mass transfer problems. In microbead pellicular anion-exchange column packings, small functionalized latex beads (<0.1-µm diameter) are attached to larger (<10-µm diameter) uniform nonporous beads. The carbohydrate analyte interacts with the functional groups at the surface of the latex microbeads, eliminating diffusion into and out of pores and the associated peak broadening.

PAD is the method of choice for the detection of carbohydrates in HPAEC because it relies on the high-pH solutions that HPAEC provides by default. Amperometric detection measures the current, or charge, resulting from the oxidation or reduction of analyte molecules at the surface of a working electrode. Electrons are transferred from the electroactive analyte to the electrode during oxidation reactions and in the opposite direction during reduction reactions. This process allows sensitive and highly selective detection of analytes that can be oxidized or reduced, but interfering species that are not electroactive remain undetected. Carbohydrates are easily oxidized at gold and platinum electrodes at high pH, and the current generated is proportional to the carbohydrate concentration.

A typical amperometric detection system contains a working electrode and a reference electrode. Gold electrodes are most common for carbohydrate analysis, but oxidation products poison the electrode surface and inhibit further oxidation. Maintaining a stable, active electrode surface is accomplished by cyclical pulsing between high positive and negative potentials. This timed series of different potentials is referred to as a waveform, and repeated application of a waveform is the basis of pulsed amperometry. Different waveforms are used for different HPAEC-PAD applications and for different working electrodes: disposable gold electrodes require the use of fast, quadruple waveforms, but other gold electrodes allow a wider range of waveforms to be used without damaging the electrode surface. Disposable electrodes and fast waveforms were introduced to minimize the influence of electrode recess on the sensitivity and precision of quantitative monosaccharide applications.

FLUOROPHORE LABELING OF MONOSACCHARIDES BEFORE SEPARATION AND QUANTIFICATION

An alternative approach to the identification and quantification of monosaccharides present in a hydrolysate is to modify the monosaccharides by reductive amination with an easily detected fluorophore label that allows high-sensitivity detection and improves the chromatographic separation of monosaccharides. Essentially standard HPLC equipment can be used and, because the same labeling approaches are applicable to cleaved oligosaccharides, a consistent analytical approach can be applied. Fluorophore labeling has been much less widely used than HPAEC-PAD for monosaccharide identification and quantification. Labeling of sialic acid derivatives is usually undertaken with 1,2-phenylenediamine (or DMB, the 4,5-methylenedioxy derivatives), and the resulting products are separated on a C-18 column and using fluorescence detection.

CONCLUSION

Because of the complexity of glycoprotein glycan structures and their inherent variation during production processes, manufacturers are generally required by means of characterization studies to develop criteria for the control of the glycosylation pattern of a biological drug substance when glycosylation occurs, as well as to develop the level of information required at each stage of production and at batch release. Then analytical procedures can be derived in a manner that provides information relevant to fulfilling quality requirements. In general, a combination of approaches and techniques is needed, and more detailed glycan structural analysis at early drug development stages is required. Validation considerations are central as method development and product knowledge progress.

APPENDIX 1

Abbreviations

Fuc	L-Fucose
Gal	D-Galactose
GalNAc	*N*-Acetyl-D-galactosamine
Glc	D-Glucose
GlcNAc	*N*-Acetyl-D-glucosamine
Man	D-Mannose
Neu5Ac	*N*-Acetylneuraminic acid
Neu5Gc	*N*-Glycolylneuraminic acid
Xyl	D-Xylose

Additional Definitions

High mannose—Glycan chains containing two core GlcNAc residues and between five and nine Man residues, and lacking Gal, GlcNAc, or Neu5Ac residues in the antennae. Such chains are typically found in mammalian glycans.

Hypermannosylation—(i) Addition of Man residues to high mannose chains creating chains with large numbers of Man residues, and (ii) *O*-Man linked glycan chains with multiple Man residues synthesized by yeast.

Paucimannose—Glycan chains containing two core GlcNAc residues between two and four Man residues. Core-linked Fucα1,3 and/or Fucα1,6 residues may be present.

Oligomannose—Used here as a generic term to include high mannose, paucimannose, and *N*-linked hyper-mannosylated chains.

⟨1086⟩ IMPURITIES IN DRUG SUBSTANCES AND DRUG PRODUCTS

INTRODUCTION

This general information chapter is intended to provide common terminology for impurities and degradation products that may be present in compendial drug substances and drug products. Impurities or degradation products in drug substances can arise during the manufacturing process or during storage of the drug substance. The degradation products in drug products can arise from drug substances or reaction products of the drug substance with the environment, with an excipient, or an immediate container–closure system. Biological and biotechnological products, fermentation products and semisynthetic products derived therefrom, and radiopharmaceutical products are not covered in this chapter.

Communications about impurities and degradation products in compendial articles may be improved by including in this Pharmacopeia the definitions of terms and the contexts in which these terms are used. (See *Glossary* below.) There has been much activity and discussion in recent years about the definition of terms. Certain industry-wide concerns about terminology and context deserve widespread publication and ready retrievability and are included here. See section *5.60, Impurities and Foreign Substances* in section *5, Monograph Components* under *General Notices and Requirements*, as well as the general chapter *Ordinary Impurities* ⟨466⟩. Some other general chapters added over the years have also addressed topics of purity or impurity as these have come into focus or as analytical methodology has become available. Analytical aspects are enlarged upon in the chapter *Validation of Compendial Procedures* ⟨1225⟩.

Purity or impurity measurements for drug products present a challenge to Pharmacopeial standards-setting. Where degradation of a drug product over time is at issue, the same analytical methods that are stability-indicating are also purity-indicating. Resolution of the active ingredient(s) from the excipients necessary to the preparation presents the same qualitative problem. Thus, many monographs for Pharmacopeial preparations feature chromatographic assays. Where more significant impurities are known, some monographs set forth specific limit tests. In general, however, this Pharmacopeia does not repeat impurity tests in subsequent preparations where these appear in the monographs of drug substances and where these impurities are not expected to increase. It is presumed that adequate retention specimens are in storage for the exact batch of drug substances used in any specific lot of a drug product. Whenever analysis of an official article raises a question of the official attributes of any of the drug substances used, subsequent analysis of retention specimens is in order.

DRUG SUBSTANCE

Classification of Impurities

Impurities can be classified into the following categories:

1. Organic impurities (process- and drug-related)

2. Inorganic impurities
3. Residual solvents

Organic impurities can arise during the manufacturing process and/or storage of the drug substance. They can be identified or unidentified, volatile or nonvolatile, and include the following:

1. Starting materials
2. Byproducts
3. Intermediates
4. Degradation products
5. Reagents, ligands, and catalysts
6. Geometric and stereoisomers

Inorganic impurities can result from the manufacturing process. They are normally known and identified and include the following:

1. Reagents, ligands, and catalysts
2. Heavy metals or other residual metals
3. Inorganic salts
4. Other materials (e.g. filter aids, charcoal)

Residual solvents are organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of a drug substance. Because these are generally of known toxicity, the selection of appropriate controls is easily accomplished (see *Residual Solvents* ⟨467⟩).

Concepts for setting impurity or degradation product limits in drug substances are based on chemistry and safety concerns. As such, limits for organic and inorganic impurities and residual solvents should be established for drug substances. The basic tenet for setting limits is that levels of impurities or degradation products in a drug substance must be controlled throughout its development to ensure its safety and quality for use in a drug product.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

DRUG PRODUCT

The specification for a drug product should include a list of degradation products expected to occur during manufacture of the commercial product and under recommended storage conditions. Stability studies, knowledge of degradation pathways, product development studies, and laboratory studies should be used to characterize the degradation profile. The selection of degradation products in the drug product specification should be based on the degradation products found in batches manufactured by the proposed commercial process.

This rationale should include a discussion of the degradation profiles observed in the safety and clinical development batches and in stability studies, together with a consideration of the degradation profile of batches manufactured by the proposed commercial process. For degradation products known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation/detection limit of the analytical procedures should be commensurate with the level at which the degradation products should be controlled.

For drug products the concept for setting degradation product limits is based on sound scientific judgment as applied to available data on the safety and stability of the drug product, data that may include the degradation pathways of the drug substance, the manufacturing process, known excipient interactions, any safety assessment studies, stability studies conducted under the recommended storage conditions, and ancillary studies that may provide additional information on the stability profile of the drug product. Impurities that are not degradation products (e.g., process impurities from the drug substance) are often not controlled in the drug product, as they are typically controlled in the drug substance and these impurities are not expected to increase over time. Additional guidance for setting limits can be found in various ICH and FDA guidance documents, as well as in the USP monograph submission guidelines.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

Drug products should contain levels of residual solvents no higher than can be supported by safety data (see *Residual Solvents* ⟨467⟩).

Change to read:

GLOSSARY

Concomitant components: Concomitant components are characteristic of many drug substances and are not considered to be impurities in the Pharmacopeial sense. Limits on contents, or specified ranges, or defined mixtures are set forth for concomitant components in this Pharmacopeia. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

Degradation product: An impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and/or the immediate container–closure system.

Foreign substances (extraneous contaminants): An impurity that arises from any source extraneous to the manufacturing process and that is introduced by contamination or adulteration. These impurities cannot be anticipated when monograph tests and assays are selected. The presence of objectionable foreign substances not revealed by monograph tests and assays constitutes a variance from the official standard. Examples of foreign substances include ephedrine in Ipecac or a pesticide in an oral liquid analgesic. Allowance is made in this Pharmacopeia for the detection of foreign substances by unofficial methods. (See section *5.60, Impurities and Foreign Substances* in section *5, Monograph Components* under *General Notices and Requirements.*)

Identified impurities and identified degradation products: Impurities or degradation products for which structural characterizations have been achieved.

Impurity: Any component of a drug substance that is not the chemical entity defined as the drug substance and in addition, for a drug product, any component that is not a formulation ingredient.

Inorganic impurities: Inorganic impurities can result from the manufacturing process (e.g., residual metals, inorganic salts, filter aids, etc.). Inorganic impurities are typically controlled by tests such as ●● (Official 1-Jan-2018) *Residue on Ignition* ⟨281⟩. Information found in *Plasma Spectrochemistry* ⟨730⟩ and *Ion Chromatography* ⟨1065⟩ may also be of value.

Intermediate: A material that is produced during steps of the synthesis of a drug substance and that undergoes further chemical transformation before it becomes a drug substance. The intermediate is often isolated during the process.

Ordinary impurities: Some monographs make reference to ordinary impurities. For more details see *Ordinary Impurities* ⟨466⟩.

Other impurities: See section *5. Monograph Components* under *General Notices and Requirements.*

Polymorphs: Different crystalline forms of the same drug substance. These can include solvation or hydration products (also knows as pseudopolymorphs) and amorphous forms. Although polymorphs are not impurities by definition, an understanding of the crystalline forms, hydration or solvation states, or amorphous nature is critical to the overall characterization of the drug substance.

Process contaminants: Process contaminants are identified or unidentified substances (excluding related substances and water), including reagents, catalysts, other inorganic impurities (e.g., heavy metals, chloride, or sulfate); and may also include foreign substances (extraneous contaminants). These contaminants may be introduced during manufacturing or handling procedures.

Reagent: A substance other than a starting material, intermediate, or solvent that is used in the manufacture of a drug substance.

Related substances: Related substances are structurally related to a drug substance. These substances may be (a) identified or unidentified impurities arising from the synthesis manufacturing process, such as starting materials, intermediates, or by-products, and do not increase on storage, or (b) identified or unidentified degradation products that result from drug substance or drug product manufacturing processes or arise during storage of a material.

Residual solvents: An organic liquid used as a vehicle for the preparation of solutions or suspensions in the synthesis of a drug substance (see *Residual Solvents* ⟨467⟩).

Specified impurities and specified degradation products: Previously referred to as Signal Impurities, specified impurities or specified degradation products are impurities or degradation products that are individually listed and limited with specific acceptance criteria in individual monographs as applicable. Specified impurities or specified degradation products can be identified or unidentified.

Starting material: A material that is used in the synthesis of a drug substance and is incorporated as an element into the structure of an intermediate and/or of the drug substance. Starting materials are often commercially available and have well-defined chemical and physical properties and structure.

Stereomeric impurity: A compound with the same 2-dimensional chemical structure as the drug substance but differs in the 3-dimensional orientation of substituents at chiral centers within that structure. In those cases where all chiral centers are in the opposite orientation, the impurity is an enantiomer (enantiomeric impurity). Determinations of impurities in this category often require special chiral chromatographic approaches. Diastereomeric or epimeric impurities occur when only some of the chiral centers are present in the opposite orientation.

Toxic impurities: Toxic impurities have significant undesirable biological activity, even as minor components, and require individual identification and quantification by specific tests. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. Based on validation data, individualized tests and specifications are selected. These feature comparison to a Reference Standard of the impurity, if available. It is incumbent on the manufacturer to provide data that would support the classification of such impurities as toxic impurities.

Unidentified impurities and unidentified degradation products: Impurities or degradation products for which structural characterizations have not been achieved and that are identified solely by qualitative analytical properties (e.g., chromatographic retention times).

Unspecified impurities and unspecified degradation products: Impurities or degradation products that are limited by general acceptance criteria but not individually listed with their own specific acceptance criteria in individual monographs.

⟨1087⟩ APPARENT INTRINSIC DISSOLUTION—DISSOLUTION TESTING PROCEDURES FOR ROTATING DISK AND STATIONARY DISK

This general information chapter *Apparent Intrinsic Dissolution—Dissolution Testing Procedures for Rotating Disk and Stationary Disk* ⟨1087⟩ discusses the determination of dissolution rates from nondisintegrating compacts exposing a fixed surface area to a given solvent medium. Compact, as used here, is a nondisintegrating mass resulting from compression of the material under test using appropriate pressure conditions. A single surface having specified physical dimensions is presented for dissolution. Determination of the rate of dissolution can be important during the course of development of new chemical entities because it sometimes permits prediction of potential bioavailability problems and may also be useful for characterizing compendial articles such as excipients or drug substances. Intrinsic dissolution studies are characterization studies and are not referenced in individual monographs. Information provided in this general information chapter is intended to be adapted via a specific protocol appropriate to a specified material.

Dissolution rate generally is expressed as the mass of solute appearing in the dissolution medium per unit time (e.g., mass sec^{-1}), but dissolution flux is expressed as the rate per unit area (e.g., mass cm^{-2} sec^{-1}). Reporting dissolution flux is preferred because it is normalized for surface area and, for a pure drug substance, is commonly called intrinsic dissolution rate. Dissolution rate is influenced by intrinsic solid-state properties such as crystalline state, including polymorphs and solvates, as well as degree of noncrystallinity. Numerous investigational approaches are available for modifying the physicochemical properties of chemical entities so that their solubility and dissolution properties are enhanced. Among these approaches are the use of coprecipitates and the use of amorphous solid dispersions. The effect of impurities associated with a material can also significantly alter its dissolution properties. Dissolution properties are also influenced by extrinsic factors such as surface area, hydrodynamics, and dissolution medium properties, including solvent (typically water), presence of surfactants, temperature, fluid viscosity, pH, buffer type, and buffer strength.

Rotating disk and stationary disk dissolution procedures are sufficiently versatile to allow study of the characteristics of compounds of pharmaceutical interest under a variety of test conditions. Characteristics common to both apparatuses include the following:

(1) They are adaptable to use with standard dissolution testing stations, and both use a tablet die to hold the nondisintegrating compact during the dissolution test.
(2) They rely on compression of the test compound into a compact that does not flake or fall free during the dissolution test.
(3) A single surface of known geometry and physical dimension is presented for dissolution.
(4) The die is located at a fixed position in the vessel, which decreases the variation of hydrodynamic conditions.

A difference between the two procedures is the source of fluid flow over the dissolving surface. In the case of the rotating disk procedure, fluid flow is generated by the rotation of the die in a quiescent fluid, but fluid flow is generated by a paddle or other stirring device for the stationary disk procedure.

EXPERIMENTAL PROCEDURE

The procedure for carrying out dissolution studies with the two types of apparatus consists of preparing a nondisintegrating compact of material using a suitable compaction device, placing the compact and surrounding die assembly in a suitable dissolution medium, subjecting the compact to the desired hydrodynamics near the compact surface, and measuring the amount of dissolved solute as a function of time.

Compacts are typically prepared using an apparatus that consists of a die, an upper punch, and a lower surface plate fabricated out of hardened steel or other material that allows the compression of material into a nondisintegrating compact. An alternative compaction apparatus consists of a die and two punches. Other configurations that achieve a nondisintegrating compact of constant surface area also may be used. The nondisintegrating compact typically has a diameter of 0.2–1.5 cm.

Compact Preparation

Attach the smooth lower surface plate to the underside of the die, or alternatively, insert the lower punch using an appropriate clamping system. Accurately weigh a quantity of material necessary to achieve an acceptable compact, and transfer to the die cavity. Place the upper punch into the die cavity, and compress the powder on a hydraulic press at a compression pressure required to form a nondisintegrating compact that will remain in the die assembly for the length of the test. Compression for 1 minute at 15 MPa usually is sufficient for many organic crystalline compounds, but alternative compression conditions that avoid the formation of capillaries should be evaluated. For a given substance, the compact preparation, once optimized is standardized to facilitate comparison of different samples of the substance.

Changes in crystalline form may occur during compression; therefore, confirmation of a solid state form should be performed by powder X-ray diffraction or other techniques. Remove the surface plate or lower punch. Remove loose powder from the surface of the compact and die by blowing compressed air or nitrogen over the surface.

Dissolution Medium

The choice of dissolution medium is an important consideration. Whenever possible, testing should be performed under sink conditions to avoid artificially retarding the dissolution rate due to approach of solute saturation of the medium. Dissolution measurements are typically made in aqueous media. To approximate in vivo conditions, measurements may be run in the physiological pH range at 37°. The procedure when possible is carried out under the same conditions that are used to determine the intrinsic solubility of the solid state form being tested. Dissolution media should be deaerated immediately before use to avoid air bubbles forming on the compact or die surface.[1]

The medium temperature and pH must be controlled, especially when dealing with ionizable compounds and salts. In the latter cases, the dissolution rate may depend strongly on the pH, buffer species, and buffer concentration. A simplifying assumption in constant surface area dissolution testing is that the pH at the surface of the dissolving compact is the same as the pH of the bulk dissolution medium. For nonionizable compounds, this is relatively simple because no significant pH dependence on dissolution rate is expected. For acids and bases, the solute can alter the pH at and near the surface of the compact as it dissolves. Under these conditions, the pH at the surface of the compact may be quite different from the bulk pH due to the self-buffering capacity of the solute. To assess intrinsic solubility, experimental conditions should be chosen to eliminate the effect of solute buffering, alteration of solution pH, and precipitation of other solid state forms at the surface of the compact. For weak acids, the pH of the dissolution medium should be 1–2 pH units below the pKa of the dissolving species. For weak bases, the pH of the dissolution medium should be 1–2 pH units above the pKa of the dissolving species.

Apparatus

ROTATING DISK

A typical apparatus (*Figure 1*) consists of a punch and die fabricated out of hardened steel. The base of the die has three threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compacted pellet. The die has a cavity into which is placed a measured amount of the material whose intrinsic dissolution rate is to be determined. The punch is then inserted in the die cavity and the test material is compressed with a hydraulic press. [NOTE—A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test.] A compacted pellet of the material is formed in the cavity with a single face of defined area exposed on the bottom of the die.

[1] One method of deaeration is the following: Heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter of 0.45-µm or less pore size, with vigorous stirring, and continue stirring under vacuum for about 5 minutes. Other deaeration techniques for removal of dissolved gases may be used.

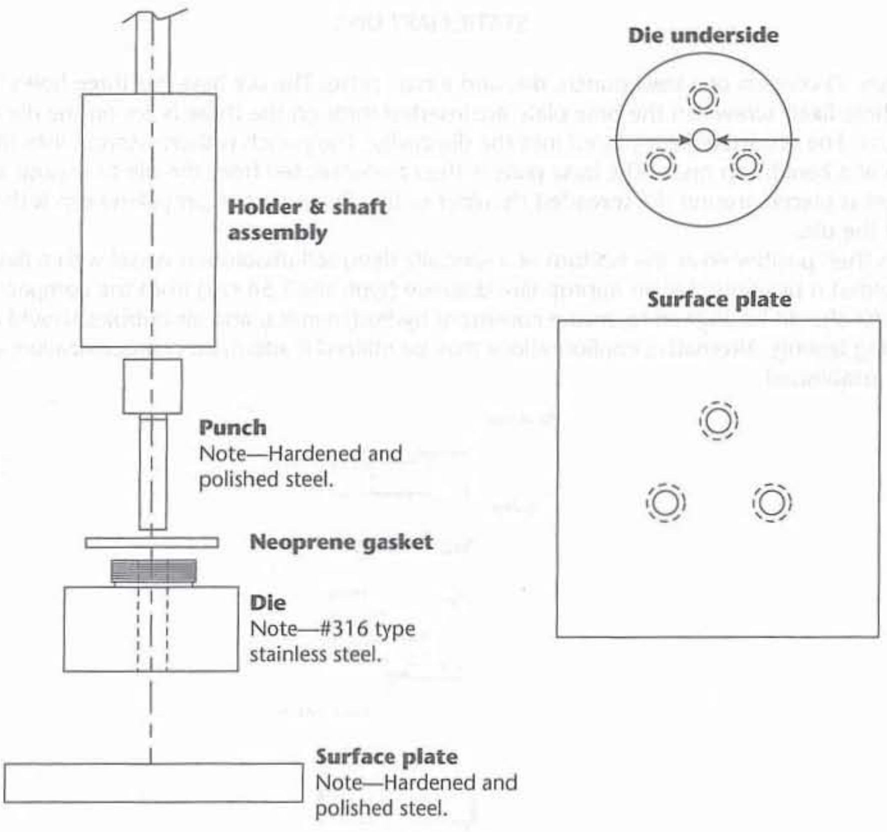


Figure 1

The die assembly is then attached to a shaft constructed of an appropriate material (typically steel). The shaft holding the die assembly is positioned so that when the die assembly is lowered into the dissolution medium (*Figure 2*), the exposed surface of the compact will be not less than 1.0 cm from the bottom of the vessel and nominally in a horizontal position. The die assembly should be aligned to minimize wobble, and air bubbles should not be allowed to form on the compact or die surface.

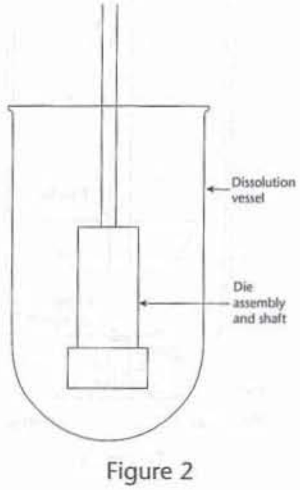


Figure 2

A rotating disk speed of 300 rpm is recommended. Typical rotation speeds may range from 60–500 rpm. The dissolution rate depends on the rotation speed used. This parameter should be selected in order to admit at least five sample points during the test, but excessive stirring speeds may create shear patterns on the surface of the dissolving material that could cause aberrant results (i.e., nonlinearity). Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process. If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

STATIONARY DISK

The apparatus (*Figure 3*) consists of a steel punch, die, and a base plate. The die base has three holes for the attachment of the base plate. The three fixed screws on the base plate are inserted through the three holes on the die and then fastened with three washers and nuts. The test material is placed into the die cavity. The punch is then inserted into the cavity and compressed, with the aid of a bench top press. The base plate is then disconnected from the die to expose a smooth compact pellet surface. A gasket is placed around the threaded shoulder of the die and a polypropylene cap is then screwed onto the threaded shoulder of the die.

The die assembly is then positioned at the bottom of a specially designed dissolution vessel with a flat bottom (*Figure 4*). The stirring unit (e.g., paddle) is positioned at an appropriate distance (typically 2.54 cm) from the compact surface. The die assembly and stirring unit should be aligned to ensure consistent hydrodynamics, and air bubbles should not be present on the compact surface during testing. Alternative configurations may be utilized if adequate characterization and control of the hydrodynamics can be established.

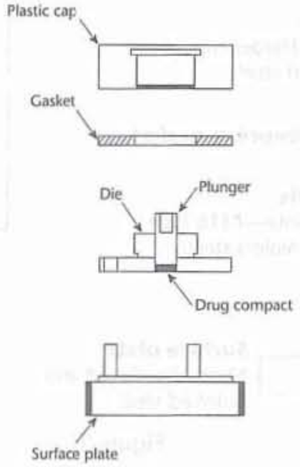


Figure 3

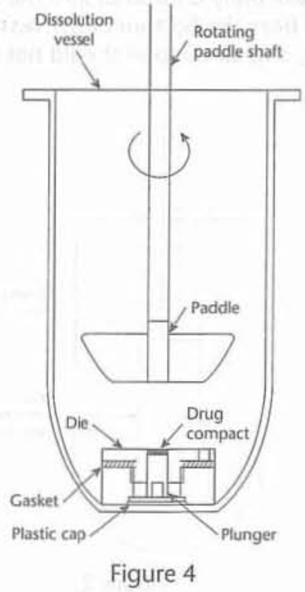


Figure 4

The dissolution rate depends on the rotation speed and precise hydrodynamics that exist. Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process (see *Rotating Disk*). If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

DATA ANALYSIS AND INTERPRETATION

The dissolution rate is determined by plotting the cumulative amount of solute dissolved against time. Linear regression analysis is performed on data points in the initial linear region of the dissolution curve. The slope corresponds to the dissolu-

tion rate (mass sec^{-1}). (More precise estimates of slope can be obtained using a generalized linear model that takes into account correlations among the measurements of the cumulative amounts dissolved at the various sampling times.)

The amount versus time profiles may show curvature. When this occurs, only the initial linear portion of the profile is used to determine the dissolution rate. Upward curvature (positive second derivative) of the concentration versus time data is typically indicative of a systematic experimental problem. Possible problems include physical degradation of the compact by cracking, delaminating, or disintegration. Downward (negative second derivative) curvature of the dissolution profile is often indicative of a transformation of the solid form of the compact at the surface or when saturation of the dissolution medium is inadvertently being approached. This often occurs when a less thermodynamically stable crystalline form converts to a more stable form. Examples include conversion from an amorphous form to a crystalline form or from an anhydrous form to a hydrate form, or the formation of a salt or a salt converting to the corresponding free acid or free base. If such curvature is observed, the crystalline form of the compact may be assessed by removing it from the medium and examining it by powder X-ray diffraction or another similar technique to determine if the exposed surface area is changing.

The constant surface area dissolution rate is reported in units of mass sec^{-1}, and the dissolution flux is reported in units of mass cm^{-2} sec^{-1}. The dissolution flux is calculated by dividing the dissolution rate by the surface area of the compact. Test conditions, typically a description of the apparatus, rotation speed, temperature, buffer species and strength, pH, and ionic strength should also be reported with the analyses.

⟨1088⟩ IN VITRO AND IN VIVO EVALUATION OF DOSAGE FORMS

PURPOSE

This chapter provides an overview of the methodology for characterizing the physicochemical properties of a drug substance as well as its associated drug product and discusses the relationship of these methods and properties to the pharmacokinetic and pharmacodynamic properties of the drug product. Results of in vitro methods are linked with information from in vivo evaluations through an in vitro–in vivo correlation (IVIVC).

SCOPE

The ultimate goal of these characterization studies is an understanding of the relationship between the physicochemical and pharmacological properties of the drug substance to the pharmacokinetic properties and in vitro performance of the drug product. This chapter outlines the in vitro and in vivo testing that goes into the development of the body of data that informs decision making relating to the formulation, manufacturing, and related regulatory activities necessary for the development, regulatory approval, and marketing of any drug product. The chapter complements the information in general chapters, *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩ and *The Dissolution Procedure: Development and Validation* ⟨1092⟩ by detailing the essential in vitro and in vivo data elements underlying an understanding of bioequivalence and bioavailability. The chapter text recognizes that regulatory guidances and a wealth of text books are available to elaborate on the content provided, and it is not the purpose to provide an exhaustive disquisition on the subjects presented but rather to provide a guide and listing of the issues of interest.

BACKGROUND INFORMATION

Establishing a meaningful relationship between dissolution behavior and in vivo drug performance (i.e., IVIVC) has long been sought from the perspectives of both bioavailability (BA) and bioequivalence (BE) and quality control considerations. In setting dissolution acceptance criteria for a product monograph, USP's policy has been to give predominant consideration to valid BA or BE studies, when available.

The earliest achievable in vitro characteristic thought to predict an acceptable in vivo performance was tablet and capsule disintegration. A test for disintegration was adopted in *USP XIV* (1950). At that time, no quantitative work was done to attempt to demonstrate such a relationship, especially with regard to in vivo product performance. Advances in instrumental methods and analytical precision ultimately opened up prospects for this work. The USP–NF Joint Panel on Physiologic Availability recognized that the disintegration test was insufficiently sensitive and in 1968 directed the identification of candidate articles for the first 12 official dissolution tests that used *Apparatus 1*.

USP requires drug release testing via the USP performance test in the majority of monographs for non-solution oral, sublingual, and transdermal dosage forms. In the current state of science, in vivo testing is necessary during the development and evaluation of both immediate-release and modified-release dosage forms. In some cases, depending on the Biopharmaceutics Classification System (BCS) classification of the drug, and depending on regulatory policy, in vivo testing may not be necessary. The special sensitivity of the dissolution test to changes in composition or method of manufacturing that do not result in significant changes in performance in vivo is well recognized. An understanding of the full complement of information given

by in vitro and in vivo evaluation of the drug substance and product is the starting point in the development of a meaningful in vitro performance test.

IN VITRO EVALUATION

Physicochemical Properties—Drug Substance

Physicochemical information typically includes polymorphism, stability, particle size distribution, solubility, dissolution rate, lipophilicity, permeability, and other release-controlling variables of the drug substance under conditions that may mimic the extremes of the physiologic environment experienced by the dosage form.

Physicochemical Properties—Drug Product

The variables tested to characterize the physicochemical properties of the drug product should be the same as those that are tested to characterize the drug substance. Dissolution profiles over a relevant pH range, usually from pH 1–6.8, should be obtained with particular attention to formulation effects. Characterization of formulations that are insoluble in aqueous systems may require the addition of sodium lauryl sulfate or another surfactant. The BCS classification of the drug substance should be determined, especially for immediate-release dosage forms.

Dissolution Testing

Dissolution testing is required for all non-solution oral, including sublingual, Pharmacopeial dosage forms in which absorption of the drug is necessary for the product to exert the desired therapeutic effect. Exceptions include tablets that meet a requirement for completeness of solution, products that contain radiolabeled drugs, or products that contain a soluble drug and demonstrate rapid (10–15 min) disintegration. Dissolution testing should be conducted on equipment that conforms to the requirements in *Dissolution* ⟨711⟩ and on which a performance verification test has been conducted when one is available. On its website, USP provides a guidance for optimizing dissolution instrument performance by mechanical calibration and performance verification testing (http://www.usp.org/pdf/EN/dissolutionProcedureToolkit2010-03.pdf).

In vitro dissolution testing generally should attempt to mimic in vivo dissolution, but such in vitro conditions cannot be selected reliably a priori. A range of in vitro dissolution test conditions (e.g., media of varying pH, surfactant, and apparatus rotational speed) should be evaluated. Knowledge of drug substance properties, product formulation, gastrointestinal physiology, in vitro dissolution, and in vivo pharmacokinetics will aid in the selection of in vitro dissolution test conditions and specifications.

For products that contain more than a single active ingredient, dissolution typically should be determined for each active ingredient. When a dissolution test is added to an existing monograph, the disintegration test is deleted, but in the case of sublingual preparations and orally disintegrating tablets, disintegration may be a critical quality attribute in addition to dissolution. In such cases one or both tests can be included in the monograph.

When a single set of specifications cannot be established for multisource products described in monographs, multiple dissolution tests are allowed, and labeling is required to indicate the appropriate dissolution test for the specific product.

Detailed information about method development and validation can be found in *The Dissolution Procedure: Development and Validation* ⟨1092⟩.

IMMEDIATE-RELEASE DOSAGE FORMS

For immediate-release dosage forms the in vitro dissolution process typically requires no more than 60 min, and in most cases a single time-point specification is adequate for Pharmacopeial purposes. To allow for typical disintegration times, test times of less than 30 min should be based on demonstrated need.

EXTENDED-RELEASE DOSAGE FORMS

For extended-release products in vivo dissolution generally is rate limiting, which results in protracted drug absorption and thus facilitates the identification of in vitro test conditions that may be predictive of in vivo dissolution. Multiple sampling time points, therefore, are necessary to define a dissolution profile for a modified-release dosage form.

The choice of apparatus should be based on knowledge of the formulation and actual dosage form performance in the in vitro test system. *Apparatus 1* (basket) or *Apparatus 2* (paddle) may be more useful at higher rotation rates (e.g., the paddle at 100 rpm). *Apparatus 3* (reciprocating cylinder) has been especially useful for bead-type modified-release dosage forms. *Apparatus 4* (flow cell) may offer advantages for modified-release dosage forms that contain active ingredients that have limited solubility. *Apparatus 7* (reciprocating disk) is applicable to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms. *Apparatus 5* (paddle over disk) and *Apparatus 6* (cylinder) also are useful for evaluating and testing transdermal dosage forms.

At least three timepoints are chosen to characterize the in vitro drug release profile of an extended-release dosage form for Pharmacopeial purposes. Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug.

IN VIVO EVALUATION OF DOSAGE FORMS

In evaluating a drug product's performance, analysts fundamentally must ask what type of study should be performed to give reasonable assurance of BE of a marketed product to the clinical trial product that demonstrated safety and efficacy. Although they provide important information concerning the release characteristics of the drug from the dosage form, in vitro dissolution studies at present are used primarily for setting or supporting specifications for drug products (e.g., shelf life) and manufacturing process control (e.g., scale-up or postapproval changes). Normally BE is best demonstrated by in vivo evaluation but can sometimes be replaced by in vitro studies.[1] BE assessment of modified-release dosage forms is best achieved by observing in vivo drug pharmacokinetic and/or pharmacodynamic behavior by means of well-designed clinical studies. Multiple guidances for the conduct of such studies are provided by regulatory agencies. Moreover, when a well-defined, predictive relationship exists between plasma concentrations of a drug or its active metabolites and the clinical response (therapeutic and adverse), it is possible to use plasma drug concentration data alone as a basis for the approval of a modified-release dosage form that is designed to replace an immediate-release dosage form.

Although human pharmacokinetic studies often are used to assess BE of immediate-release solid oral dosage forms, in some cases in vitro studies can be used to assess BE. The principal advantage of in vitro studies is that they reduce development costs. For example, an in vitro test is preferable when one is testing BCS Class I drugs with rapid dissolution. Some regulatory agencies permit this type of testing in lieu of in vivo testing.

The following discussions are intended to provide guidance for drug substance evaluation and the design, conduct, and evaluation of studies involving dosage forms. Although these guidelines focus on oral drug delivery systems, the principles may be applicable to other routes of drug administration (e.g., transdermal, subcutaneous, intramuscular, etc.).

CHARACTERIZATION OF DRUG SUBSTANCE

The Biopharmaceutics Classification System (BCS)

FDA has issued a guidance titled "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070246.pdf). A key assumption in the approach is that drug release and dissolution is sufficiently rapid so that an in vitro–in vivo correlation is not possible and/or useful. When applicable, the BCS allows dissolution rate data in lieu of BA or BE studies for product approval.

Pharmacokinetic Properties

Analysts should thoroughly characterize the input absorption profile of the active drug entity from a formulation that shows rapid BA (an intravenous solution, oral solution, or a well-characterized immediate-release drug product). In turn, this formulation serves as a reference to evaluate the input profile of the modified-release dosage form. This information, together with the pharmacokinetics of the active drug entity, can characterize drug absorption and can predict changes in drug BA when input is modified as in modified-release dosage forms. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism, a reduction in systemic availability could result after oral administration if the input rate is decreased.

In designing an oral modified-release dosage form, analysts may find it useful to determine the absorption of the active drug entity in various segments of the gastrointestinal tract, particularly in the lower gastrointestinal tract (colon) for delayed-release dosage forms that release drug in this region. Food effects also may be important and should be investigated.

Drug Disposition

The information required to characterize drug disposition may include the following.

1. Disposition parameters—clearance, area under the time—plasma concentration curve (AUC), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), volume of distribution, half-life, mean residence time, or model-dependent parameters.
2. Linearity or characterization of nonlinearity over the dose or concentration range that could be encountered.
3. Drug/metabolite accumulation.
4. Metabolic profile and excretory pathway, with special attention to the active metabolites and active enantiomers of racemic mixtures.

[1] 21 CFR 320.22 Criteria for waiver of evidence of in vivo bioavailability or bioequivalence.

General Chapters

5. Enterohepatic circulation.
6. Protein-binding parameters and effect of dialysis.
7. The effects of age, gender, race, and relevant disease states.
8. Plasma: blood ratios.
9. A narrow therapeutic index or a clinical response that varies significantly as a function of the time of day (chronopharmacokinetics).

Pharmacodynamic Properties

Before developing a dosage form, analysts should obtain concentration–response relationships over a dose range sufficiently wide to encompass important therapeutic and adverse responses. In addition, the equilibration-time[2] characteristics between plasma concentration and effect should be evaluated. For modified-release products that typically have larger drug doses in the dosage form, these concentration–response relationships should be sufficiently characterized so that a reasonable prediction of the safety margin can be made if dose dumping should occur. If there is a well-defined relationship between the plasma concentration of the active drug substance or active metabolites and the clinical response (therapeutic and adverse), the clinical performance of a new modified-release dosage form could be characterized by plasma concentration–time data. If such data are not available, clinical trials of the modified-release dosage form should be carried out with concurrent pharmacokinetic and pharmacodynamic measurements.

CHARACTERIZATION OF THE DOSAGE FORM

Pharmacokinetic Properties: Immediate-Release Products

The types of pharmacokinetic studies that should be conducted are based on how much is known about the active drug substance, its clinical pharmacokinetics, and its BCS Class. For example, a new chemical entity requires greater pharmacokinetic characterization than does an FDA-approved formulation that is undergoing scale-up and postapproval changes (SUPAC) evaluation.

The latter is seen when an FDA-approved drug product undergoes changes in the manufacturing of the product after the product has been approved. Such changes are common and can be caused by expansion in the size of the lots manufactured, new manufacturing locations, or the introduction of new technology. Necessary in vitro dissolution tests and/or in vivo BE tests are described in the FDA "Guidance for Industry: Immediate-release Solid Oral Dosage Forms: Scale-up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070636.pdf).

Similar requirements apply to a generic equivalent of an approved immediate-release dosage form that must be BE to the innovator drug, known as the reference listed drug. The two most frequently used methods for meeting bioequivalence requirements are in vivo pharmacokinetic studies and BCS-based in vitro studies.

Pharmacokinetic Properties: Modified-Release Products

Like the approaches for immediate-release products, the types of pharmacokinetic studies that should be conducted for modified-release products are based on how much is known about the drug substance, its pharmacokinetics, biopharmaceutics, and whether pharmacokinetic studies are intended to be the sole basis for product approval. At a minimum, two studies are required to characterize the product when no reference modified-release product exists: (1) a single-dose crossover study for each strength of a modified-release dosage form and (2) a multiple-dose, steady-state study using the highest strength of a modified-release dosage form. A food effects study to evaluate the potential for dose dumping from extended-release dosage forms also is required as a separate study or is included as an arm of a crossover study. In the demonstration of interchangeability, a single-dose, fasting crossover study vs. the reference product usually will suffice. In some cases, a food-effects study is required if the reference product has demonstrated a food effect on BA. Some appropriate single-dose crossover and multiple-dose steady-state studies are described below.

For modified-release products, intravenous solutions, oral solutions, or well-characterized immediate-release drug products are possible reference products to evaluate a modified-release formulation. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism from the small intestine, a reduction in systemic availability could result after oral administration if the input rate is decreased. An increase in systemic availability could be observed if a drug is absorbed from the colon from a delayed-release dosage form that targets the colon, thus avoiding a first-pass effect.

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[2] Equilibration time is a measure of the time-dependent discontinuity between measured plasma concentrations and measured effects. The discontinuity is more often characterized by the degree of hysteresis observed when the effect-concentration plot for increasing concentrations is compared with that for decreasing concentrations. Where the equilibration time is very short (i.e., rapid equilibration with no active metabolites generated), there will be little or no hysteresis. That is, the same effect will be observed for a given concentration independent of the interval between the time of dosing and the time that measurements are made.

In some modified-release capsule dosage forms, the strengths differ from each other only in the amount of identical beaded material contained in each capsule. In this case, single-dose and multiple-dose steady-state studies at the highest dosage strength are sufficient. Other strengths can be characterized on the basis of comparative in vitro dissolution data.

The pharmacokinetic studies described below are needed for most modified-release dosage forms. These studies may be the basis for characterization of the dosage form. If regulatory approval is sought without conducting clinical trials, manufacturers should consult with the regulatory authorities to ensure that an adequate database exists for the approval. The types of pharmacokinetic studies generally conducted can be categorized as follows.

CASE A

Case A applies to an original modified-release oral dosage form for a drug already marketed in an immediate-release dosage form and for which extensive pharmacokinetic/pharmacodynamic data exist.

Single-dose crossover study: A single-dose crossover study should include the following treatments: the modified-release dosage form administered under fasting conditions; a dosage form that is rapidly available administered under fasting conditions; and the modified-release dosage form administered immediately after a high-fat standardized meal. The food effects study should control the ambient-temperature fluid intake (e.g., 6–8 oz.) at the time of drug administration. The dosage form should be administered within 5 min after completion of the meal. Ideally all subjects should consume the meal in approximately 15 min. If there are no significant differences in the rate or extent of bioavailability (AUC, C_{max} and T_{max}) as a function of the meal, then additional food effect studies are not necessary. If significant differences in bioavailability are found, researchers must define how food affects the modified-release dosage form,[3] as well as how the food–drug effect relates to time.

Use the following guidelines in evaluating food effects.

1. If no well-controlled studies have previously defined the effects of a concurrent high-fat meal on an immediate-release dosage form, studies should be performed to determine whether a food effect is a result of problems with the dosage form. Does the dosage form show food-related changes in release, or are there food effects that are unrelated to the dosage form, e.g., changes in the drug's absorption from the gastrointestinal tract or changes in the drug's disposition that are independent of absorption? The cause of the food effect should be determined by a single-dose crossover study comparing the solution (or immediate-release dosage form) under fed and fasting conditions. If there is no food effect, then one concludes that there are problems with the dosage form. If there is a food effect, then one concludes that the effect is unrelated to the dosage form.
2. The influence of timing on the food effect should be tested by a four-way crossover study, in which the modified-release dosage form is administered under the following treatment conditions: fasting, taken with a high-fat meal, 1 h before a high-fat meal, and 2 h after a high-fat meal.
3. If the food effect on an immediate-release dosage form is determined to result from changes in the dissolved drug's absorption from the gastrointestinal tract or from changes in drug disposition, studies should define the appropriate relationship between drug dosing and meals.
4. Alternative appropriate studies can be conducted if the applicant labels the drug for administration with a meal that is not fat loaded. In this case, an alternative meal composition should be considered.
5. Analysts should monitor the entire single-dose, modified-release absorption profile. Where appropriate (e.g., in a multiple-dose study) for specific drugs and drug delivery systems, blood samples should be taken following breakfast on the second day, before the second dose is administered. This sampling schedule is particularly important for once-a-day products.
6. For delayed-release (enteric-coated) dosage forms, analysts should perform BA studies to characterize food effect and to support the dosing claims stated in the labeling.

The purpose of these studies is twofold: first, to determine whether a need exists for labeling instructions describing special conditions for administration with respect to meals; and second, to provide information concerning the pattern of absorption of the modified-release dosage form compared to that of the immediate-release dosage form. Drug input function should be defined for modified-release dosage forms. This will aid in the development of an appropriate in vitro dissolution test. For dosage forms that exhibit high variability, a replicate study design is recommended.

Multiple-dose, steady-state studies

Study I—When data demonstrating linear pharmacokinetics exist for an immediate-release dosage form, a steady-state study should be conducted with the modified-release dosage form at one dose rate (preferably at the high end of the usual dosage regimen) using a comparable total daily dose of an immediate-release dosage form as a control. At least three trough plasma drug concentration (C_{min}) determinations at the same time of day should be made to demonstrate that steady-state conditions have been achieved. Plasma drug concentration determinations, over at least one dosing interval of the modified-release dosage form, should be made in each phase of the crossover study. It may be preferable (as in the case of rhythmic variation in absorption or disposition of the drug) to measure concentrations over an entire day in each phase. The presence or absence of circadian variation should be verified. The modified-release dosage form should produce an AUC that is equivalent to that of the immediate-release dosage form if the extent of absorption from the modified-release dosage form is comparable to the immediate-release dose. The degree of fluctuation for the modified-release product should be the same as, or less than, that

[3] Wagner–Nelson, Loo–Riegelman, and other deconvolution methods are found in textbooks on biopharmaceutics.

General Chapters

for the immediate-release dosage form given by the approved regimen. Appropriate concentration measurements should include unchanged drug and major active metabolites. For racemic drug entities, analysts should consider measurement of the active enantiomers.

Study II—When comparisons of the pharmacokinetic properties of an immediate-release dosage form at different doses are not available, or when the data demonstrate nonlinearity, steady-state crossover studies comparing effects of the modified-release dosage form and those of the immediate-release dosage form should be conducted at two different dose rates: one at the low end of the recommended dosing range and the second at the high end of the dosing range. In each case, the modified-release dosage form must meet the criteria described in *Study I* with respect to AUC and fluctuations in plasma drug concentrations. If there are significant differences between the modified-release dosage form and the immediate-release dosage form at either the low or the high dosing rate, these data alone are not adequate to characterize the product. Data can be misleading when obtained from subjects with atypical drug disposition or physiologic characteristics relative to the target population. Therefore, subject selection should be from an appropriate target population with randomized assignment to dosage form population. If the modified-release dosage form is for use in a specific subpopulation (e.g., for children), it should be tested in that population. Whether a drug exhibits linear or nonlinear pharmacokinetics, the basis for characterization is equivalence of AUC and of the relative degree of fluctuation of concentrations of the modified-release and immediate-release dosage forms.

Steady-state studies in selected patient populations or drug interaction studies may also be necessary, depending on the therapeutic use of the drug and the types of individuals for whom the modified-release dosage form will be recommended. For drugs that have narrow therapeutic indices, it may be necessary to perform more extensive plasma concentration measurements to determine the potential for unusual drug-release patterns in certain subpopulations. In such studies, researchers should perform more than one AUC measurement per patient to assess variability with both the modified-release and the immediate-release dosage forms.

General Chapters

CASE B

Case B applies to a non-oral, modified-release dosage form of an already marketed active drug entity for which extensive pharmacokinetic and pharmacodynamic data exist.

Case A studies (omitting the food effects studies) are appropriate for the evaluation of a modified-release dosage form designed for a non-oral route of administration if the pattern of biotransformation to active metabolites is identical for the two routes. If the biotransformation patterns are different, then clinical efficacy studies should be performed with the modified-release dosage form. In addition, special studies may be necessary to assess specific risk factors related to the dosage form (e.g., irritation and/or sensitization at the site of application of a transdermal drug delivery system).

CASE C

Case C applies to a generic equivalent of an approved modified-release dosage form, which should be BE to the reference drug in its rate and extent of drug exposure (i.e., AUC, C_{max}, C_{min}, and degree of fluctuation) in crossover single-dose studies. For an oral modified-release dosage form, the food studies described under *Case A* also should be performed.

CASE D

Case D applies to an FDA-approved product that has undergone SUPAC. Necessary in vitro dissolution tests and/or in vivo bioequivalence tests are described in the FDA guidance, *SUPAC-MR: Modified Release Solid Oral Dosage Forms; Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation* (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070640.pdf).

Statistical Analysis of In Vivo Bioequivalence

An appropriate statistical method should be selected. (See *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* ⟨1090⟩).

IN VITRO–IN VIVO CORRELATIONS

The term IVIVC first appeared in the pharmaceutical literature as a result of the awareness of the importance of bioavailability concepts and in vitro dissolution rate determinations. IVIVC refers to the establishment of a rational relationship between a biological property, or a parameter derived from drug plasma concentrations produced by a dosage form, and a physicochemical property or characteristic of the same dosage form. The biological properties most commonly used are one or more pharmacokinetic parameters such as C_{max} or AUC, obtained following the administration of the dosage form. The physicochemical property most commonly used is a dosage form's in vitro dissolution behavior (e.g., percent of drug released under a given set of conditions). The quantitative relationship between the two properties, biological and physicochemical, is an IVIVC. The

most important use of an IVIVC is for predictability. In many cases the actual drug plasma concentration profile can be predicted from in vitro dissolution data.

Historically, IVIVC analysis has been more successful for extended-release products than for immediate-release products. This difference probably reflects the application of specific data analysis techniques and interpretations that require dissolution rate–limited drug absorption. However some correlations with immediate-release products have been demonstrated using methods that rely on the current, broad availability of computers and nonlinear regression software, along with new correlation methods.

General Considerations

With the proliferation of modified-release products, it becomes necessary to examine IVIVC in greater detail. Unlike immediate-release dosage forms, modified-release products, particularly extended-release dosage forms, cannot be characterized using a single time point dissolution test. These products are designed to deliver drug so that a patient has a specific plasma level profile over a prolonged period, usually 12–24 h. Analysts require an in vitro means of ensuring that each batch of the product will perform identically in vivo. An IVIVC satisfies this requirement. Initially, it was thought that developing a meaningful correlation for immediate-release dosage forms would be an easier task than for extended-release products. However, because of the nature of the principles on which each type is based, analysts now believe that an IVIVC is more readily achieved for modified-release dosage forms.

One expects all extended-release products to be dissolution rate limited. For these products, the formulation significantly contributes to the prolongation of drug release from the dosage form. Because of the impact of formulation on BA from an extended-release product, numerous attempts have been made to correlate one or more pharmacokinetic parameters determined from in vivo studies with the amount released in a given time during an in vitro dissolution test. Single-point correlations can indicate that increasing or decreasing the in vitro dissolution rate of the modified-release dosage form would result in a corresponding directional change in the product's performance. However, such single-point correlations reveal little about the overall plasma level curve, which is a major factor for drug performance in the patient. Rather, correlation methods that utilize all plasma drug concentration data and all in vitro dissolution data are preferred. Three correlation procedures are available that use all dissolution and plasma data, along with statistical moment analysis. Each procedure displays important differences in the quality of the correlation. These methods are discussed in terms of the advantages of each along with its potential utility as a predictive tool for pharmaceutical scientists.

Correlation Levels

Three correlation levels have been defined and categorized in descending order of quality. The concept of correlation level is based on the ability of the correlation to reflect the entire plasma drug concentration–time curve that results from administration of the given dosage form. The relationship of the entire in vitro dissolution curve to the entire plasma concentration–time profile defines the strength of the correlation and, therefore, the predictability.

LEVEL A

This level is the highest category of correlation. It represents a point-to-point relationship between in vitro dissolution and the in vivo input rate (absorption rate of the drug from the dosage form). For a *Level A* correlation, a product's in vitro dissolution curve is compared to its in vivo input curve, i.e., the curve produced by deconvolution of the plasma profile. Deconvolution can be accomplished using mass balance model-dependent methods, such as the Wagner–Nelson or Loo–Riegelman methods, or by model-independent, mathematical deconvolution. In an ideal correlation, the in vitro dissolution and in vivo absorption rate curves are superimposable or can be made superimposed by the use of a constant offset value of the time scale. The equations describing each curve are the same. This procedure often is found with modified-release dosage systems that demonstrate an in vitro release rate that is essentially independent of the dissolution media and stirring speeds used in a dissolution apparatus. Superimposition is not an absolute requirement for a *Level A* correlation. If the dissolution and absorption curves are different and a mathematical relationship can be developed to relate the two, the plasma level profile still is predictable from the in vitro dissolution data. This relationship must be true not only at that single input rate but also over the entire quality control dissolution range for the product. Furthermore, when the dissolution rate depends on mixing speed, the two curves can be made to superimpose by either increasing or decreasing the in vitro mixing speed or some other alteration of the dissolution method.

The advantages of a *Level A* correlation are as follows.

1. It develops a point-to-point correlation. This is not found with any other correlation level. It is developed using every plasma level and dissolution point collected at different time intervals, so it reflects the complete plasma level curve. As a result, in the case of a *Level A* correlation an in vitro dissolution curve can serve as a surrogate for in vivo performance. A

change in manufacturing site, method of manufacture, raw material supplies, minor formulation modifications, and even product strength using the same formulation can be justified without the need for additional BA-BE studies.[4,5]

2. A truly meaningful quality control procedure that indicates in vivo performance and is predictive of a dosage form's performance is defined for the dosage form.
3. The extremes of the in vitro quality control standards can be justified either by convolution (simulating the plasma level profile from the dissolution curve) or by deconvolution (using the upper and lower confidence interval limits).

LEVEL B

This correlation uses the principles of statistical moment analysis. The mean in vitro dissolution time is compared to either the mean residence time or the mean in vivo dissolution time. As with a *Level A* correlation, *Level B* uses all of the in vitro and in vivo data but is not considered a point-to-point correlation. It does not correlate the actual in vivo plasma profiles but rather a parameter that results from statistical moment analysis of a plasma profile component such as mean residence time. Because a number of different plasma profiles can produce similar mean residence time values, one cannot rely on a *Level B* correlation alone to predict a plasma profile from in vitro dissolution data. In addition, in vitro data from such a correlation cannot be used to justify values at the extremes of quality control standards.

LEVEL C

This category relates one dissolution time point ($t_{50\%}$, $t_{90\%}$, etc.) to one pharmacokinetic parameter such as AUC, C_{max}, or T_{max}. It represents a single-point correlation and does not reflect the complete shape of the plasma profile, which best defines the performance of modified-release products. Because this type of correlation is not predictive of actual in vivo product performance, generally it is useful only as a guide in formulation development or as a production quality control procedure. Because of its obvious limitations, a *Level C* correlation has limited usefulness in predicting in vivo drug performance and is subject to the same caveats as a *Level B* correlation in its ability to support product and site changes as well as justification of the extreme values in quality control standards. The FDA Guidance "Extended-Release Solid Oral Dosage Forms—Development, Evaluation, and Application of In Vitro/In Vivo Correlations" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070239.pdf) states that manufacturers can obtain biowaivers based on multiple *Level C* correlations. The guidance shows how manufacturers can achieve this correlation. The FDA also indicates that if such a correlation is achievable, it is likely that the development of a *Level A* correlation is also feasible.

DEVELOPING A CORRELATION

This chapter does not define the only procedures for developing an IVIVC, and any well-designed and scientifically valid approach is acceptable. To assist the pharmaceutical scientist, one possible procedure for developing a *Level A* correlation is described below:

1. In order to perform deconvolution properly, analysts should be familiar with the pharmacokinetics of the drug itself as well as when it is incorporated into a modified-release dosage form. For example, if a drug is known to be fully absorbed but demonstrates saturable first-pass kinetics, it is best to assume 100% bioavailability for purposes of absorption rate calculation. This is based upon the fact that the drug is fully absorbed, but because of liver metabolism, one sees less than if the drug were administered as an immediate-release bolus. If one utilizes the extent of absorption relative to an immediate-release or solution dosage form, the input profiles will not superimpose with that calculated assuming 100% absorption. However, point-to-point correlations most likely will be possible.
2. Different dissolution profiles of a formulation should be obtained as illustrated in *Figure 1*. The formulation should be modified only sufficiently to produce different dissolution profiles so that the formulation has the same excipients in all the lots that will be tested. The formulation modifications used in these batches should be based on factors that would be expected to influence the product's modified-release rate and could occur during normal product manufacture. In vitro drug release is performed on the batches that will be used in the bioavailability study, and the effect of varying the dissolution conditions is investigated. Some of the variables that should be studied are the apparatus (it is preferable to use official dissolution equipment), mixing intensity, and dissolution medium (i.e., pH value, enzymes, surfactants, osmotic pressure, ionic strength, etc.). The dissolution behavior of the dosage form need not be studied under all of the conditions indicated. The number of conditions investigated depends largely on whether a correlation can be developed with the in vitro results obtained under the more commonly investigated conditions such as apparatus, agitation intensity, or dissolution medium and pH value. Each formulation and every drug represents an individual challenge. The resulting dis-

[4] FDA Guidance SUPAC-MR: Modified Release Solid Oral Dosage Forms—Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation (1997).

[5] FDA Guidance Extended-Release Solid Oral Dosage Form—Development, Evaluation, and Application of In Vitro/In Vivo Correlations, "If an IVIVC is developed with the highest strength, waivers for changes made on the highest strength and any lower strengths may be granted if these strengths are compositionally proportional or qualitatively the same, the in vitro dissolution profiles of all the strengths are similar, and all strengths have the same release mechanism."

solution profiles from the use of different dissolution media are illustrated in *Figures 1* and *2* in which the same formulations were tested in water and an acid buffer.

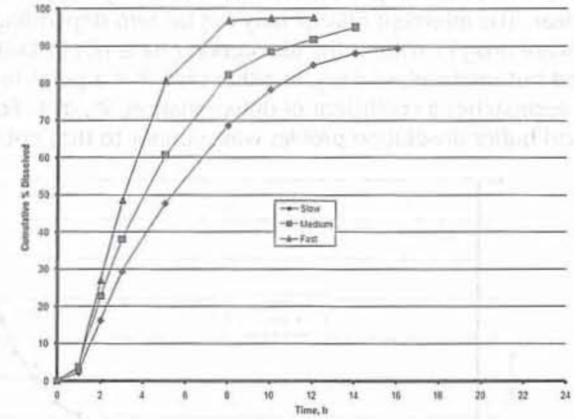


Figure 1. Mean dissolution profiles of three modifications of a new modified-release formulation (USP Apparatus 2, 50 rpm, 0.9 L water, 37°).

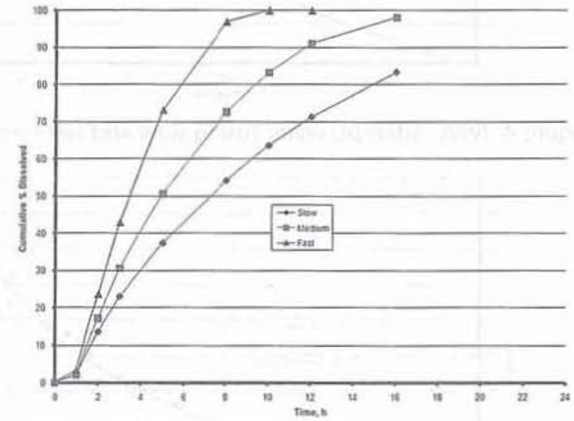


Figure 2. Mean dissolution profiles of a new modified-release formulation (USP Apparatus 2, 50 rpm, 0.9 L, pH 4.5 buffer, 37°).

3. The plasma level or urinary excretion data obtained in the definitive bioavailability study of the modified-release dosage form are treated by a deconvolution procedure. The resulting data may represent the drug input rate of the dosage form. They also represent in vivo dissolution when the rate-controlling step of the dosage form is its dissolution rate (i.e., drug absorption after dissolution is considered to be instantaneous). Any deconvolution procedure (e.g., mass balance or mathematical deconvolution) will produce acceptable results. *Figure 3* illustrates the results of numerical deconvolution of the plasma profiles obtained for the batches in *Figures 1* and *2*.

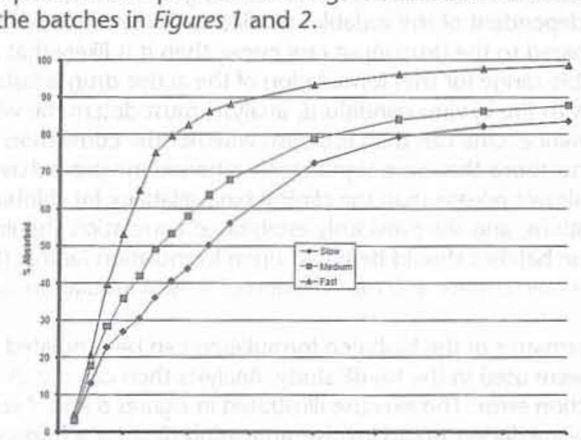


Figure 3. Mean absorption profiles from numerical deconvolution of plasma concentration–time plots.

4. The in vitro dissolution curve is then compared to the drug absorption rate curve. This can be performed by various methods. Simply positioning one curve on the other often can indicate the existence of a correlation. This may then be

quantified by defining the equation for each curve and comparing the corresponding constants. The simplest way to demonstrate a correlation is to plot the fraction absorbed in vivo vs. the fraction released in vitro, as illustrated in *Figures 4* and *5*. With a *Level A* correlation, this relationship is often linear with a slope approaching 1. As illustrated in *Figures 4* and *5*, a correlation may be curvilinear. The intercept may or may not be zero depending upon whether there is a lag time before the system begins to release drug in vivo, or the absorption rate is not instantaneous, resulting in the presence of some finite quantity of dissolved but unabsorbed drug. In either case, it is a point-to-point or a *Level A* correlation when the least-squares fit of the line approaches a coefficient of determination, R^2, of 1. For the correlations illustrated in *Figures 4* and *5*, the IVIVC using the acid buffer dissolution profiles was superior to that obtained from water.

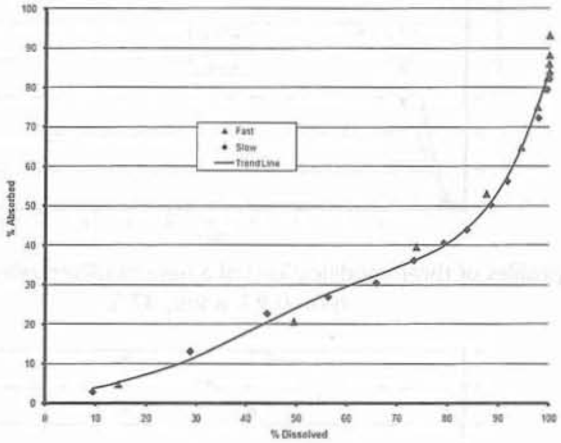


Figure 4. IVIVC attempt: water (using slow and fast formulations).

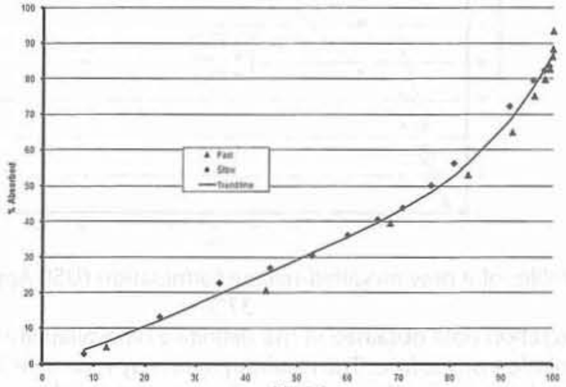


Figure 5. IVIVC attempt: pH 4.5 buffer.

5. If from the studies indicated in the in vitro dissolution evaluation, given above, the modified-release dosage form exhibits dissolution behavior that is independent of the variables studied and a *Level A* correlation is demonstrated when the in vitro dissolution curve is compared to the drug input rate curve, then it is likely that the correlation is general and can be extrapolated within a reasonable range for that formulation of the active drug substance. If the dosage form exhibits dissolution behavior that varies with the in vitro conditions, analysts must determine which set of dissolution conditions best correlates with in vivo performance. One can then establish whether the correlation is real or an artifact. This is achieved by preparing at least two formulations that have significantly different in vitro behavior. One should demonstrate a more rapid release and the other a slower release than the clinical bioavailability lot (biobatch). A pilot BA-BE study should be performed with these formulations, and the previously established correlation should be demonstrated for both. The formulation modifications of these batches should be based upon formulation factors that would be expected to influence the product's modified-release mechanism, and modification of these formulation factors are expected to influence the dosage form's release rate.
6. Alternatively, the in vivo performance of the biobatch formulation can be simulated based on the correlation developed with these formulations that were used in the BA-BE study. Analysts then can compare the predicted and experimentally determined values, the prediction error. The exercise illustrated in *Figures 6* and *7* serves as an internal validation of the *Level A* correlation. An external validation would involve simulating data for a formulation batch that was not included in the *Level A* correlation calculations. Such a validation was performed using the in vivo data from the medium lot of the formulation, and the results are illustrated in *Figure 8*.

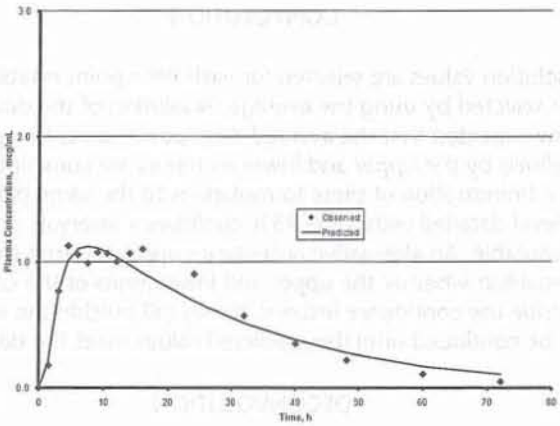


Figure 6. Observed and predicted mean plasma profiles: slow formulation.

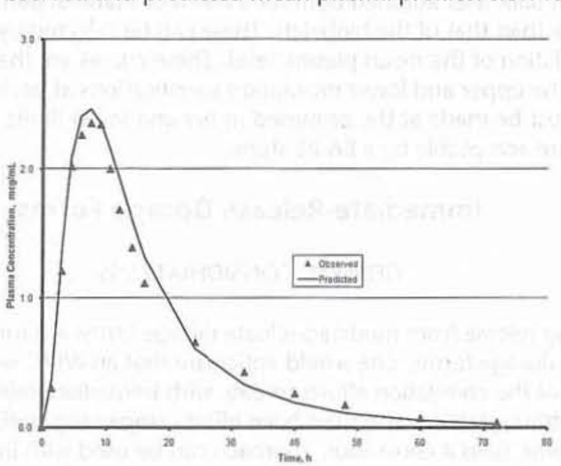


Figure 7. Observed and predicted mean plasma profiles: fast formulation.

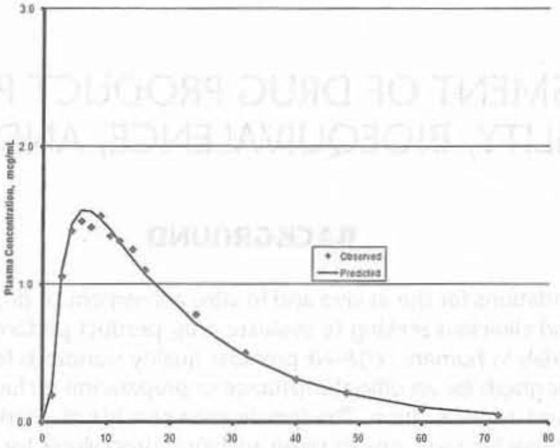


Figure 8. Observed and predicted mean plasma profiles: medium formulation.

7. Once a *Level A* correlation is established, in vitro testing can be used to establish dissolution specifications, biowaivers to facilitate SUPAC, and changes in dosage form strength for the same formulation. It is questionable whether such an extrapolation with *Level B* and C correlations is possible.

Establishment of Dissolution Specification Ranges

It is relatively easy to establish a multipoint dissolution specification for a modified-release dosage form. The dissolution behavior of the biobatch can be used to define the amount that will be released at each time point. The difficulty arises in the variation that will be allowed around each time point. In the case of a *Level A* correlation, this can be done in two ways, both of which use IVIVC: convolution and deconvolution.

CONVOLUTION

Reasonable upper and lower dissolution values are selected for each time point established from the biobatch. Historically, dissolution specifications have been selected by using the average dissolution of the development batches, with a range of ±2.5–3 standard deviations. It is now expected that the average dissolution values be approximately the same as those of the biobatch. The dissolution curves defined by the upper and lower extremes are convoluted to project the anticipated plasma level curves that would result from administration of these formulations to the same patients to whom the biobatch was administered. If the resulting plasma level data fall within the 95% confidence intervals obtained in the definitive BA-BE study, these ranges can be considered acceptable. An alternative acceptance approach that can be used after the therapeutic window for a drug has been defined, is to establish whether the upper and lower limits of the convolution results fall within the therapeutic window, even if they fall outside the confidence interval. If they fall outside the window, a more limited range must be established. This procedure should be continued until the predicted values meet the desired ranges.

DECONVOLUTION

An acceptable set of plasma-level data is established both for a batch of material demonstrating a more rapid release and for one demonstrating a slower release than that of the biobatch. These can be selected by using the extremes of the 95% confidence intervals or ±1 standard deviation of the mean plasma level. These curves are then deconvoluted, and the resulting input rate curve is used to establish the upper and lower dissolution specifications at each time point. In the case of *Level B* and *C* correlations, batches of product must be made at the proposed upper and lower limits of the dissolution range, and it must be demonstrated that these batches are acceptable by a BA-BE study.

Immediate-Release Dosage Forms

GENERAL CONSIDERATIONS

Because the mechanisms for drug release from modified-release dosage forms are more complex and variable than those associated with immediate-release dosage forms, one would anticipate that an IVIVC would be easier to develop with the latter formulations. Unfortunately, most of the correlation efforts to date with immediate-release dosage forms have been based on the correlation *Level C* approach, although there also have been efforts employing statistical moment theory (*Level B*). Although it is conceivable that the same *Level A* correlation approach can be used with immediate-release dosage forms, until data have been gathered to support this concept, *Level B* and *Level C* are the best approaches that can be recommended with these dosage forms.

⟨1090⟩ ASSESSMENT OF DRUG PRODUCT PERFORMANCE—BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

BACKGROUND

This chapter provides recommendations for the in vivo and in vitro assessment of drug product performance. The chapter is intended as a guide to scientists and clinicians seeking to evaluate drug product performance by surrogate procedures correlative and/or antecedent to clinical trials in humans. *USP–NF* provides quality standards for drug substances, excipients, and finished preparations. A *USP–NF* monograph for an official substance or preparation includes the article's definition; packaging, storage, and other requirements; and a specification. The specification consists of a series of universal tests (description, identification, impurities, and assay) and specific tests, one or more analytical procedures for each test, and acceptance criteria. Quality standards are important attributes that must be built into the drug product. Meeting *USP–NF* standards is accepted globally as assurance of high quality and is part of the requirements necessary for approval of a bioequivalent (BE), interchangeable multisource drug product. Multisource drug products must meet certain in vivo and/or in vitro performance standards to be considered therapeutically equivalent and interchangeable. Regulatory approval for interchangeable multisource products may differ somewhat in each country (see the forthcoming chapter *Essentials for Drug Product Selection* ⟨1096⟩ for further discussion). Drug product performance may be defined as the release of the active pharmaceutical ingredient (API) from the drug product dosage form, leading to systemic availability of the API necessary for achieving a desired therapeutic response. This chapter discusses in vivo and in vitro approaches to determining drug product performance. The focus of the chapter is primarily on the performance of solid oral drug products.

The chapter references a Food and Drug Administration (FDA) guidance, *Guidance for Industry—Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General Considerations* (2003) (http://www.fda.gov/; search by document

title) and a World Health Organization (WHO) document titled *Annex 7 Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability* (2006) (http://who.int/en/; search by document title). FDA guidances are used in the United States; and WHO, FDA, and national/regional guidelines may be used by national/regional drug regulatory authorities. Following approval, control of the quality of a drug product can be achieved in part by the private and/or public specification, which can include a performance test. USP provides the general chapters *Disintegration* ⟨701⟩, *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩, and *The Dissolution Procedure: Development and Validation* ⟨1092⟩, which describe these tests and procedures.

This chapter provides general information about the conduct of bioequivalence (BE) studies as a surrogate measure of in vivo drug product performance and dissolution profile comparisons as a measure of in vitro drug product performance. The chapter also discusses conditions when an in vivo BE requirement may be waived (biowaiver) for certain drug products and shows how the Biopharmaceutics Classification System (BCS) can be used as a predictor of a drug product's performance. An appendix to this chapter defines key scientific terminology and provides a comparison between FDA and WHO in drug product performance assessment.

BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

Bioavailability (BA) studies focus on determining the process and time frame by which a drug is released from the oral dosage form and moves to the site of action [see FDA Guidance *Guidance for Industry—Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General Considerations* (2003)]. BA is an indirect or surrogate measure of the rate and extent to which the API or active moiety is absorbed from a drug product and becomes available at its target sites of action. BA data provide an estimate of systemic drug exposure, including fraction of drug absorbed. For drug products that are not intended to be absorbed into the bloodstream, availability may be assessed by measurements that reflect the rate and extent to which the active ingredient or active moiety becomes available at the sites of action. Drug products are considered BE if a test drug product does not show a significant difference in rate and extent of absorption by comparison with a designated reference drug when administered at the same molar dose of the same active moiety in the same dosage form under similar experimental conditions in either a single dose or in multiple doses.

BA and BE generally can be obtained by serially measuring drug and/or metabolite concentrations in the systemic circulation over a prescribed period. BE studies can use other approaches when systemic drug concentrations cannot be measured or are not appropriate. For these cases, more indirect approaches to BE determination include acute pharmacodynamic endpoints, clinical endpoints, and in vitro studies that typically involve comparisons of the dissolution profiles of test and reference drug products.

BA and BE information are important in regulatory submissions. BA information broadly addresses the absorption, distribution, metabolism, and excretion of the API. For an innovator product, BE studies establish the performance of the product intended for marketing by comparing the bioavailability of the product as developed for marketing approval to the clinical trial material, the drug product used in safety/efficacy trials. For the development and regulatory approval of a generic drug product, the test drug product must be BE to the reference listed drug (RLD) product (usually the brand or innovator drug product that is designated by the applicable regulatory authority).

The ICH document titled *Guidance on Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* (2000) (http://www.fda.gov/; search by document title) provides approaches for setting acceptance criteria for drug product performance. This approach relies on dissolution or disintegration based on clinically acceptable batches, as does FDA's. BE studies focus on the performance of the drug product and usually involve comparisons of two drug products: the test (T) and reference (R) or comparator product. The required studies and the determination of BE are the province of regulatory agencies. In the United States, R is termed the *reference listed drug* (RLD) and is so noted in FDA's *Approved Drug Products with Therapeutic Equivalence Ratings* [*Orange Book* (2008) (http://www.fda.gov/cder/ob)]. To assist countries and regions where the R product may not always be readily identifiable, WHO has prepared a document titled *Annex 11 Guidance on the Selection of Comparator Pharmaceutical Products for Equivalence Assessment of Interchangeable Multisource (Generic) Products* (2005) (http://www.who.int/en/; search by document title). In the WHO document, R is termed the *comparator pharmaceutical product* (CPP). When a country or region has a clearly defined set of CPPs, the task becomes one of requiring that a manufacturer demonstrate, to the satisfaction of its regulatory authority, that its multisource product is pharmaceutically equivalent and BE to the corresponding CPP.

Bioequivalence

An interchangeable multisource (generic) product must be pharmaceutically equivalent (PE). The WHO document allows pharmaceutical alternatives to be considered therapeutically equivalent and interchangeable if they are BE. Further, generic products must be shown to be BE in order to be considered therapeutically equivalent (TE) to the R product (CPP). For the product to be considered PE, it must have the same active ingredient, same strength, same dosage form, same route of administration, and same labeling as the comparator product. Several methods exist to assess and document BE. These include the following:

1. *Comparative pharmacokinetic studies in humans.* In these studies, the active drug and/or its metabolite(s) are measured as a function of time in accessible biological fluid such as blood, plasma, serum, or urine to obtain pharmacokinetic measures

General Chapters

such as area under the plasma drug concentration vs. time curve (AUC) and maximum concentration (C_{max}) that are reflective of systemic exposure.

BE studies are designed to compare the in vivo performance of a generic product with an R product. Generally the design is a two-period, two-sequence, single-dose, crossover randomized one carried out in 18 to 36 subjects. The number of subjects should be statistically justified and not less than 12. During the study, blood samples are collected at sufficient intervals for assessing C_{max}, AUC, and other parameters. Blood samples are analyzed using appropriately validated bioanalytical methodology with standard pharmacokinetic measures and statistical approaches. The statistical method for testing pharmacokinetic BE is based on the determination of the 90% confidence interval around the geometric mean ratio of the log-transformed population means (generic/R) for AUC and C_{max} by carrying out two one-sided tests at the 5% level of significance.

2. *Other options.* In addition, comparative pharmacodynamic studies in humans and comparative clinical trials can be used to document or supplement BE assessment. Beyond these clinical studies, in vitro dissolution based on the BCS can ensure BE between T and R products. In vivo documentation of equivalence is especially important for the following: narrow therapeutic range drugs; documented evidence of BE problems; modified-release pharmaceutical products designed to act by systemic absorption; and fixed-dose combination products with systemic action when at least one of the APIs requires an in vivo study.

IMMEDIATE-RELEASE DRUG PRODUCTS

Single-dose, crossover BE studies are carried out at the highest dose comparing T and R products under fasting conditions. A parallel study design can be used for drugs that have a very long elimination half-life ($t_{1/2}$). Sampling truncation at 72 hours may be allowable by regulatory agencies. Lower strength(s) of the dosage form can be given a biowaiver based on dosage form proportionality and dissolution profile similarity. Food-effect studies are required if there is an indication in the labeling that concomitant administration of food may diminish, increase, or not influence the BA of the drug product.

MODIFIED-RELEASE DRUG PRODUCTS

BE studies for extended-release dosage forms are carried out as single-dose, crossover studies under fasting and fed conditions at the highest dose to compare T and R products. A single-dose study is more sensitive than multiple-dose, steady-state studies in assessing in vivo drug product performance, particularly with regard to the phenomenon of dose dumping, i.e., the rapid and unintended premature release of the active ingredient from an extended-release product into the bloodstream. Lower strengths of an extended-release dosage form may not require an in vivo study based on use of the same drug-releasing mechanism, dosage form proportionality, and similar dissolution profile.

ORALLY ADMINISTERED DRUG PRODUCTS, NOT FOR SYSTEMIC EFFECT

Some oral drug products are intended for local activity. Mesalamine and cholestyramine are examples of drugs that are intended for local activity. For these types of drugs, systemic absorption from the gastrointestinal tract is minimal; thus a comparative clinical trial is required while a systemic drug exposure profile also may be required. In some cases, in vitro studies may be appropriate; such as including comparison of cholestyramine binding to bile salts.

BIOEQUIVALENCE STUDIES

Objective: The objective of a BE study is to measure and compare formulation performance between two or more pharmaceutically equivalent drug products. Drug availability from T and R products should not be statistically different when the drug is administered to patients or subjects at the same molar dose under similar experimental conditions.

Design: The design of a BE study depends on the objectives of the study, the ability to analyze the drug (and metabolites) in biological fluids, the pharmacodynamics of the drug substance, the route of drug administration, and the nature of the drug and drug product. Pharmacokinetic parameters, pharmacodynamic parameters, clinical observations, and/or in vitro studies may be used to determine drug BA from a drug product.

Some possible BE study designs include the following:

1. Single-dose, two-way crossover study under fasted conditions
2. Single-dose, two-way crossover study under fed conditions
3. Single-dose, parallel study under fasted conditions
4. Single-dose, replicate design
5. Single-dose, partial replicate design
6. Multiple-dose, two-way crossover study, fasted conditions
7. Pharmacodynamic or clinical endpoint study
8. In vitro dissolution profile comparisons

The standard BE study is a crossover design (e.g., Latin square crossover design) in which each subject receives the test drug product and the reference product on separate occasions. Studies are usually evaluated by a single-dose, two-period, two-treatment, two-sequence, open-label, randomized crossover design comparing equal doses of the test and reference products in fasted or fed adult healthy subjects. A multiple-dose study may be required for some extended-release drug products. A washout period is scheduled between the two periods to allow the subjects to completely eliminate the drug absorbed from the first dose before administration of the second dose. If the predose concentration is ≤5% of the C_{max} value in that subject, the subject's data without any adjustments can be included in all pharmacokinetic measurements and calculations. Samples of an accessible biologic fluid such as blood characterize the drug concentration vs. time profile. During the fasting study subjects are fasted at least 10 hours. A pre-dose (0 time) blood sample is taken. The drug product is given with 240 mL (8 fluid ounces) of water. No food is allowed for at least 4 hours post-dose. Blood sampling is performed periodically after dose administration according to protocol. A food intervention or food effect study is conducted with standard meal conditions that are expected to provide the greatest effects on gastrointestinal physiology so that systemic drug availability is maximally affected. In addition, the high lipid content of the meal may affect the rate of drug release from the product, in situ. A high-fat (approximately 50% of total caloric content of the meal) and high-calorie (approximately 800 to 1000 calories) meal is recommended as a test meal for food-effect BA and fed BE studies. This test meal should derive approximately 150, 250, and 500–600 calories from protein, carbohydrate, and fat, respectively. The drug product is given with 240 mL (8 fluid ounces) of water after ingestion of the standard meal. Subjects should consume identical meals at the same time during a testing period.

Analysis of samples: Samples, usually plasma, are analyzed for the active drug and, on occasion, active metabolite concentrations by a validated bioanalytical method.

Pharmacokinetic parameters: Pharmacokinetic parameters are obtained from the resulting concentration-time curves. Two major pharmacokinetic parameters are used to assess the rate and extent of systemic drug absorption. AUC reflects the extent of drug absorption, and the peak drug concentration (C_{max}) reflects the rate of drug absorption. Other pharmacokinetic parameters may include the time to peak drug concentration (T_{max}), the elimination rate constant (k), elimination half-life ($t_{1/2}$), lag time (T_{lag}), and others.

STATISTICAL ANALYSIS

Pharmacokinetic parameters are analyzed statistically to determine whether the T and R products yield comparable values. Because BE studies may use small sample sizes, log transformation of the data allows the frequency distribution of the data to be more normalized so that parametric statistical analyses may be performed (FDA, *Guidance for Industry: Statistical Approaches to Establishing Bioequivalence* (2001) (http://www.fda.gov/; search by document title).

Parametric (normal-theory) general linear model procedures are recommended for the analysis of pharmacokinetic data derived from in vivo BE studies. An analysis of variance (ANOVA) should be performed on the pharmacokinetic parameters AUC and C_{max} using appropriate statistical programs and models. For example, for a conventional two-treatment, two-period, two-sequence (2 × 2) randomized crossover study design, the statistical model often includes factors accounting for the following sources of variation:

- Sequence (sometimes called Group or Order)
- Subjects, nested in sequences
- Period (or Phase)
- Treatment (sometimes called Drug or Formulation)

The sequence effect should be tested using the [subject (sequence)] mean square from the ANOVA as an error term. All other main effects should be tested against the residual error (error mean square) from the ANOVA. The least-squares means (LSMEANS) statement should be used to calculate least-squares means for treatments. Estimates should be obtained for the adjusted differences between treatment means and the standard error associated with these differences.

The statistical assumptions underlying the ANOVA are as follows:

- Randomization of samples
- Homogeneity of variances
- Additivity (linearity) of the statistical model
- Independence and normality of residuals

In BE studies, these assumptions can be interpreted as follows:

- The subjects chosen for the study should be randomly assigned to the sequences of the study.
- The variances associated with the two treatments, as well as between the sequence groups, should be equal or at least comparable.
- The main effects of the statistical model, such as subject, sequence, period, and treatment effect for a standard 2 × 2 crossover study, should be additive. There should be no interactions between these effects.
- The residuals of the model should be independently and normally distributed.

If these assumptions are not met, additional steps should be taken prior to the ANOVA, including data transformation to improve the fit of the assumptions or use of a nonparametric statistical test in place of ANOVA. However, the normality and constant variance assumptions in the ANOVA model are known to be relatively robust (i.e., a small or moderate departure from each, or both of these assumptions, will not have a significant effect on the final result). The rationale for log transforma-

tion is provided in FDA's Guidance *Statistical Approaches to Establishing Bioequivalence.* Justification should be provided if untransformed data is to be used.

The two one-sided tests procedure: A testing procedure termed the two one-sided tests procedure is used to determine the comparability of geometric mean values for pharmacokinetic parameters measured after administration of the test and reference products.[1] The two one-sided tests procedure decides whether T is not importantly less than R and whether R is not importantly less than T. Most often, 20% defines an important difference. The statistical procedure involves the calculation of a confidence interval for the ratio (or difference) between T and R pharmacokinetic variable averages. The limits of the observed confidence interval must fall within a predetermined range for the ratio (or difference) of the product averages. Point estimate mean ratios (T/R) derived from the log-transformed AUC and C_{max} data must be between 80% and 125%. Because data are log transformed, T/R = 80/100 = 80% and R/T = 100/80 = 125%. In addition, the 90% confidence intervals for the geometric mean ratios (T/R) for AUC and C_{max} must be between 80% and 125%. The regulatory requirements for the range of 90% confidence intervals for C_{max} may be different in countries outside the United States.

Bio-inequivalence: The failure to demonstrate BE may be due to a performance failure of the T product or to an inadequate study design. The failure to demonstrate BE due to an inadequate study design can be due to improper sampling in which (1) the sampling time for C_{max} was not properly obtained or (2) the number of samples taken did not adequately describe the plasma drug concentration vs. time profile. Often with highly variable drugs (e.g., %CV >30%), too few subjects were used in the study, and therefore the study was not powered adequately.

Presentation of data: The drug concentration in biological fluid at each sampling time point should be furnished untransformed for all the subjects who participated in the study. The derived pharmacokinetic parameters also should be furnished untransformed. The mean, the standard deviation, and the coefficient of variation (CV) for each variable should be computed and tabulated in the final report.

To facilitate BE comparisons, pharmacokinetic parameters for each individual should be displayed in parallel for the formulations tested. In particular, for AUC and C_{max}, the difference (T – R), the ratio (T/R), and the log of ratio (log T/R or ln T/R) between the T and R values should be tabulated side by side for all the subjects. For each subject, the summary tables should indicate in which sequence (T, R or R, T) the subject received the product. Histograms showing the frequency distribution of the difference and ln ratio (or log ratio) for the major pharmacokinetic parameters (AUC and C_{max}) are useful in the submission.

In addition to the arithmetic mean for the T and R products, the geometric means (antilog of the means of the logs), means of the logs, and standard deviations of the logs should be calculated for AUC and C_{max}. All means, including arithmetic mean, geometric mean, and means of the logs, as well as standard deviations and CVs, should be included in the report.

Dissolution and In Vitro Product Performance

As noted for an official preparation, *USP* monographs provide a public specification that includes a list of tests, references to analytical procedures, and acceptance criteria. Most solid oral dosage forms, including oral suspensions, require a dissolution or a drug release test. Drug dissolution and drug release testing are described in *USP* general chapters *Dissolution* ⟨711⟩ and *Drug Release* ⟨724⟩. These public specifications are used for quality control tests and for market approval. The *USP* dissolution test in the monograph is related to BA and BE only when closely allied with a sound regulatory determination. Without this link it should be regarded solely as a quality control test for batch release. FDA Guidances are (1) *Guidance for Industry—Dissolution Testing of Immediate Release Solid Oral Dosage Forms* (1977) (http://www.fda.gov/; search by document title) and (2) *Guidance for Industry—Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlation* (1977) (http://www.fda.gov/; search by document title).

DISSOLUTION AND IN VITRO BIOAVAILABILITY

Drug dissolution and release tests are very useful during drug product development in identifying critical manufacturing attributes such as the impact of ingredient properties and the impact of the manufacturing process on drug product performance. During product development, optimum dissolution conditions need to be developed to discriminate drug product formulations and changes in manufacturing processes. After the finished dosage form is approved for marketing, drug dissolution and release tests are useful in predicting possible changes in performance due to scale-up and postapproval changes (SUPAC). See the following FDA guidances:

Guidance for Industry—Immediate Release Solid Oral Dosage Forms, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation (1995) (http://www.fda.gov/; search by document title) and

Guidance for Industry—SUPAC-MR: Modified-Release Solid Oral Dosage Forms: Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation (1977) (http://www.fda.gov/; search by document title).

[1] Schuirmann DJ. A comparison of the two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. *J. Pharmacokinetics and Biopharmaceutics,* 1987:15:657–680.

For some oral drug products, in vitro drug dissolution can be related to in vivo performance, such as bioavailability and/or systemic drug exposure. *USP* general information chapter *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩ describes various approaches to in vitro–in vivo correlation (IVIVC).

DISSOLUTION AND IN VITRO EQUIVALENCE

The dissolution test is a powerful in vitro physiochemical test that measures drug product quality and performance for a variety of dosage forms, such as solid oral dosage forms, transdermal dosage forms, suspensions, and certain semisolid dosage forms. The *USP* tests for finished dosage forms can be divided into two types: (1) drug product quality tests and (2) drug product performance tests. Product quality tests are intended to assess attributes such as assay and content uniformity; product performance tests are designed to assess product performance and in many cases relate to dissolution. For details regarding the performance of a dissolution test, see *USP* general chapters ⟨711⟩, ⟨724⟩, ⟨1088⟩, and ⟨1092⟩.

The in vitro dissolution test was initially developed as a quality control tool to ensure drug product quality and batch-to-batch consistency. The test procedures for conducting dissolution tests are described in *USP* general chapters ⟨711⟩ and ⟨724⟩. The development of the BCS brings new understanding and power to the dissolution test. The BCS classifies the drug substance according to the solubility and the permeability of the drug through a biomembrane such as the intestinal mucosal cells. The dissolution rate of the drug from the dosage form is important in substantiating biowaivers based on the BCS.

DISSOLUTION PROFILE COMPARISONS

In vitro drug dissolution and release testing can be related to in vivo drug performance, such as BA. The comparisons of dissolution profiles are gaining importance as a means of documenting comparative BA studies—that is, BE. A biowaiver is the replacement or waivers of in vivo BE studies by an in vitro test.

A model independent mathematical approach is used to compare the dissolution profile of two products: (1) to compare the dissolution profile between the T (generic, multisource) product and R (comparator) product in biowaiver considerations; (2) to compare the dissolution profile between the two strengths of products from a given manufacturer; and (3) for SUPAC after the product is approved. For comparing the dissolution profile, the similarity factor f_2 should be computed using the equation

$$f_2 = 50 \bullet \log\left\{\left[1+(1/n)\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} \bullet 100\right\}$$

where R_t and T_t are the cumulative percentage of the drug dissolved at each of the selected n time points of the reference and test product, respectively. An f_2 value of 50 or greater (50 to 100) ensures dissolution profile similarity and the sameness or equivalence of the two curves, and thus the performance of the two products. At a minimum, three points, no more than one point exceeding 85%, should be used for similarity profile comparison. For products that dissolve very rapidly (≥85% dissolution in 15 minutes) a profile comparison is not necessary.

Biowaiver

The term *biowaiver* is applied to a regulatory approval process when the application (dossier) is approved on the basis of evidence of equivalence other than an in vivo BE test. For solid oral dosage forms, the evidence of equivalence is determined on the basis of an in vitro dissolution profile comparison between the multisource and the comparator product.

BIOWAIVER BASED ON THE PHARMACEUTICAL DOSAGE FORM

A drug product's in vivo comparative BA or BE study requirement may be waived if the products compared contain the same API(s) in the same concentration, contain the same excipients in comparable concentrations, and meet one of the following criteria:

- Aqueous solutions to be administered parenterally
- Solutions for oral use that do not contain an excipient that is known or is suspected to affect gastro-intestinal transit or absorption of the active substance
- Gases
- Powders for reconstitution as a solution
- Otic or ophthalmic products prepared as aqueous solutions
- Topical products prepared as aqueous solutions
- Inhalation products or nasal sprays tested to be administered with essentially the same device. Special in vitro performance testing should be required to document comparable device performance.

General Chapters

BIOWAIVER BASED ON DOSAGE FORM PROPORTIONALITY

When a single-dose fasting BE study is conducted on the designated (usually highest) strength of the drug product, the requirement for the conduct of additional in vivo BE studies on the lower strengths of the same product can be waived, provided that the lower strength (1) is in the same dosage form; (2) is proportionally similar in its active and inactive ingredients; (3) has the same drug release mechanism (for extended-release products); (4) meets an appropriate in vitro dissolution profile comparison criterion ($f_2 \geq 50$); and (5) both lower and higher strengths are within the linear pharmacokinetic range.

BIOWAIVER BASED ON THE BIOPHARMACEUTICS CLASSIFICATION SYSTEM

The BCS is based on aqueous solubility and intestinal permeability of the API. When the properties of the API are evaluated in conjunction with the dissolution of the pharmaceutical dosage form, the BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate-release dosage forms. On the basis of the solubility and permeability of the dosage form, the drug substance is placed in one of four classes:

Class 1: high solubility, high permeability
Class 2: low solubility, high permeability
Class 3: high solubility, low permeability
Class 4: low solubility, low permeability

Use of the BCS has become a means of documenting BE without the conduct of an in vivo study; see the FDA Guidance *Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* (2000) (http://www.fda.gov/; search by document title).

The in vitro dissolution studies are generally carried out by basket method at 100 rpm or by paddle method at 50 rpm (FDA Guidance cited immediately above) or 75 rpm [WHO Guidance, *Annex 7 Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability* (2006)] http://www.who.int/en/; search by document title) in 900 mL of medium at pH 1.2, 4.5, and 6.8. On the basis of dissolution rate, the pharmaceutical dosage forms are classified as (1) very rapidly dissolving, if 85% or greater of the dosage form dissolves in 15 minutes or less; (2) rapidly dissolving, if 85% or greater of the dosage form dissolves in 30 minutes; or (3) slowly dissolving, if the dosage form takes more than 30 minutes for 85% of drug dissolution.

For biowaiver, the dissolution tests should be carried out for both generic and reference product under the same test conditions. For the generic product to be eligible for biowaiver, the reference product should belong to the same BCS class and should meet dissolution profile comparison criteria. Based on BCS classification and dissolution profile comparison, biowaiver can be considered by regulatory authorities provided the dissolution profile similarity criteria provided in the next sections are met.

Class 1 drug products (allowed in WHO and FDA approaches): Dosage forms of drug substances that are highly soluble, highly permeable, and rapidly dissolving are eligible for biowaivers under the following conditions:

1. 85% or more of the dosage form dissolves in 30 minutes or less and the dissolution profile of the generic product is similar to that of the reference product in pH 1.2, 4.5, and 6.8 buffer, using the basket method at 100 rpm or the paddle method at 50 rpm (FDA) or 75 rpm (WHO), and meets the criterion of dissolution profile similarity, $f_2 \geq 50$.
2. If both the reference and the generic dosage forms are very rapidly dissolving (i.e., 85% dissolution in 15 minutes or less in all three media under the above test conditions), then profile determination is not necessary.

Class 2 drug products (WHO approach): Dosage forms of drug substances with high solubility only in pH 6.8 and high permeability (low solubility by definition, BCS Class 2) are eligible for biowaivers, provided that:

1. The dosage form is rapidly dissolving (85% or more in 30 minutes or less) in pH 6.8 buffer.
2. The generic product exhibits dissolution profiles similar to those of the comparator product in buffers at pH 1.2, 4.5, and 6.8.

Class 3 drug products (WHO approach): Dosage forms of drug substances that are highly soluble and have low permeability are eligible for biowaivers under the following conditions:

1. Both the reference and the generic dosage forms are very rapidly dissolving (85% dissolution in 15 minutes or less in all three media under the test conditions given above), and they do not contain any excipients and/or inactive substances that are known to alter gastrointestinal motility and/or permeability or influence drug absorption.
2. Firms should show that the quantity of excipients used is consistent with the intended use. When new excipients and/or atypically large amounts of commonly used excipients are included in the dosage form, additional information documenting the absence of any significant impact on bioavailability of the drug is required.

DISSOLUTION AS A QUALITY CONTROL TEST AND A BE TEST

There is a clear difference between dissolution as a quality control test and dissolution as an in vitro equivalence (BE) test. For immediate-release dosage forms, the quality control test involves a single-point dissolution test in only one medium (generally a compendial test). On the other hand, the in vitro equivalence test (BE test) involves dissolution profile comparison in pH 1.2, 4.5, and 6.8 between the T product and the R product.

APPENDIX

Appendix: Comparison of FDA and WHO Definitions

Term	FDA	WHO
Pharmaceutical Equivalents	Drug products are considered pharmaceutical equivalents if they contain the same active ingredient(s), are of the same dosage form, have the same route of administration, and are identical in strength or concentration. Pharmaceutically equivalent drug products are formulated to contain the same amount of active ingredient in the same dosage form and to meet the same or compendial or other applicable standards (strength, quality, purity, and identity); but they may differ in characteristics such as shape, scoring configuration, release mechanisms, packaging, excipients, expiration time, and, within certain limits, labeling.	Products are pharmaceutical equivalents if they contain the same molar amount of the same API(s) in the same dosage form; if they meet comparable standards; and if they are intended to be administered by the same route. Pharmaceutical equivalence does not necessarily imply therapeutic equivalence, because differences in the excipients and/or the manufacturing process and some other variables can lead to differences in product performance.
Pharmaceutical Alternatives	Drug products are considered pharmaceutical alternatives if they contain the same therapeutic moiety but are different salts, esters, or complexes of that moiety or are different dosage forms or strengths.	Products are pharmaceutical alternative(s) if they contain the same molar amount of the same active pharmaceutical moiety or moieties but differ in dosage form (e.g., tablets vs. capsules) and/or chemical form (e.g., different salts, different esters). Pharmaceutical alternatives deliver the same active moiety by the same route of administration but are otherwise not pharmaceutically equivalent. They may or may not be BE or TE with the comparator product.
Therapeutic Equivalents	Drug products are considered to be therapeutic equivalents only if they are pharmaceutical equivalents and if they can be expected to have the same clinical effect and safety profile when administered to patients under the conditions specified in the labeling.	Two pharmaceutical products are considered to be therapeutically equivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and after administration in the same molar dose, their effects, with respect to both efficacy and safety, are essentially the same when administered to patients by the same route under the conditions specified in the labeling.
Bioavailability (BA)	This term means the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action.	The rate and extent to which the active pharmaceutical ingredient or active moiety is absorbed from a pharmaceutical dosage form and becomes available [at the site(s) of action] in the general circulation.
Bioequivalent Drug Products (BE)	This term describes pharmaceutical equivalent or pharmaceutical alternative products that display comparable BA when studied under similar experimental conditions.	Two pharmaceutical products are BE if they are pharmaceutically equivalent or pharmaceutical alternatives and their BA, in terms of peak concentration (C_{max}), time to peak concentration (T_{max}), and total exposure (AUC) after administration of the same molar dose under the same conditions, are similar to such a degree that their effects can be expected to be essentially the same.
RLD (Reference Product) or Comparator Product	An RLD [21 CFR 314.94(a)(3)] means the listed drug identified by FDA as the drug product upon which an applicant relies in seeking approval of its ANDA.	The comparator product is a pharmaceutical product with which the multisource product is intended to be interchangeable in clinical practice. The comparator product normally will be the innovator product for which efficacy, safety, and quality have been established. The selection of the comparator product usually is made at the national level by the drug regulatory authority.
Generic Product	A generic product is a product that is therapeutically equivalent to the RLD and is intended to be interchangeable with the innovator product.	
Multisource Pharmaceutical Products		Pharmaceutically equivalent or pharmaceutically alternative products that may or may not be therapeutically equivalent. Multisource pharmaceutical products that are therapeutically equivalent are interchangeable.
Interchangeable Pharmaceutical Product		An interchangeable pharmaceutical product is one that is therapeutically equivalent to a comparator product and can be interchanged with the comparator in clinical practice.

⟨1091⟩ LABELING OF INACTIVE INGREDIENTS

This informational chapter provides guidelines for labeling of inactive ingredients present in dosage forms.

Within the past few years a number of trade associations representing pharmaceutical manufacturers have adopted voluntary guidelines for the disclosure and labeling of inactive ingredients. This is helpful to individuals who are sensitive to particular substances and who wish to identify the presence or confirm the absence of such substances in drug products. Because of the actions of these associations, the labeling of therapeutically inactive ingredients currently is deemed to constitute good pharmaceutical practice.

Although the manufacturers represented by these associations produce most of the products sold in this country, not all manufacturers, repackagers, or labelers here or abroad are members of these associations. Further, there are some differences in association guidelines. The guidelines presented here are designed to help promote consistency in labeling.

In accordance with good pharmaceutical practice, all dosage forms [NOTE—for requirements on parenteral and topical preparations, see the *General Notices*] should be labeled to state the identity of all added substances (therapeutically inactive ingredients) present therein, including colors, except that flavors and fragrances may be listed by the general term "flavor" or "fragrance." Such listing should be in alphabetical order by name and be distinguished from the identification statement of the active ingredient(s).

The name of an inactive ingredient should be taken from the current edition of one of the following reference works (in the following order of precedence): (1) the *United States Pharmacopeia* or the *National Formulary;* (2) *USAN and the USP Dictionary of Drug Names;* (3) CTFA *Cosmetic Ingredient Dictionary;* (4) *Food Chemicals Codex*. An ingredient not listed in any of the aforementioned reference works should be identified by its common or usual name (the name generally recognized by consumers or health-care professionals) or, if no common or usual name is available, by its chemical or other technical name.

An ingredient that may be, but not always is, present in a product should be qualified by words such as "or" or "may also contain."

The name of an ingredient whose identity is a trade secret may be omitted from the list if the list states "and other ingredients." For the purposes of this guideline, an ingredient is considered to be a trade secret only if its presence confers a significant competitive advantage upon its manufacturer and if its identity cannot be ascertained by the use of modern analytical technology.

An incidental trace ingredient having no functional or technical effect on the product need not be listed unless it has been demonstrated to cause sensitivity reactions or allergic responses.

Inactive ingredients should be listed on the label of a container of a product intended for sale without prescription, except that in the case of a container that is too small, such information may be contained in other labeling on or within the package.

⟨1092⟩ THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION

INTRODUCTION

Purpose

The Dissolution Procedure: Development and Validation ⟨1092⟩ provides a comprehensive approach covering items to consider for developing and validating dissolution procedures and the accompanying analytical procedures. It addresses the use of automation throughout the test and provides guidance and criteria for validation. It also addresses the treatment of the data generated and the interpretation of acceptance criteria for immediate- and modified-release solid oral dosage forms.

Scope

Chapter⟨1092⟩ addresses the development and validation of dissolution procedures, with a focus on solid oral dosage forms. Many of the concepts presented, however, may be applicable to other dosage forms and routes of administration. General recommendations are given with the understanding that modifications of the apparatus and procedures as given in *USP* general chapters need to be justified.

The organization of ⟨1092⟩ follows the sequence of actions often performed in the development and validation of a dissolution test. The sections appear in the following sequence.

1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/DISSOLUTION METHOD DEVELOPMENT)
    - 1.1 Performing Filter Compatibility
    - 1.2 Determining Solubility and Stability of Drug Substance in Various Media

1.3 Choosing a Medium and Volume
1.4 Choosing an Apparatus
2. METHOD DEVELOPMENT
2.1 Deaeration
2.2 Sinkers
2.3 Agitation
2.4 Study Design
2.4.1 Time Points
2.4.2 Observations
2.4.3 Sampling
2.4.4 Cleaning
2.5 Data Handling
2.6 Dissolution Procedure Assessment
3. ANALYTICAL FINISH
3.1 Sample Processing
3.2 Filters
3.3 Centrifugation
3.4 Analytical Procedure
3.5 Spectrophotometric Analysis
3.6 HPLC
4. AUTOMATION
4.1 Medium Preparation
4.2 Sample Introduction and Timing
4.3 Sampling and Filtration
4.4 Cleaning
4.5 Operating Software and Computation of Results
4.6 Common Deviations from the Compendia Procedures That May Require Validation
5. VALIDATION
5.1 Specificity/Placebo Interference
5.2 Linearity and Range
5.3 Accuracy/Recovery
5.4 Precision
5.4.1 Repeatability of Analysis
5.4.2 Intermediate Precision/Ruggedness
5.4.3 Reproducibility
5.5 Robustness
5.6 Stability of Standard and Sample Solutions
5.7 Considerations for Automation
6. ACCEPTANCE CRITERIA
6.1 Immediate-Release Dosage Forms
6.2 Delayed-Release Dosage Forms
6.3 Extended-Release Dosage Forms
6.4 Multiple Dissolution Tests
6.5 Interpretation of Dissolution Results
6.5.1 Immediate-Release Dosage Forms
6.5.2 Delayed-Release Dosage Forms
6.5.3 Extended-Release Dosage Forms
7. REFERENCES

1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/ DISSOLUTION METHOD DEVELOPMENT)

Before method development can begin, it is important to characterize the molecule so that the filter, medium, volume of medium, and apparatus can be chosen properly in order to evaluate the performance of the dosage form.

1.1 Performing Filter Compatibility

Filtration is a key sample-preparation step in achieving accurate test results. The purpose of filtration is to remove undissolved drug and excipients from the withdrawn solution. If not removed from the sample solution, particles of the drug will continue to dissolve and can bias the results. Therefore, filtering the dissolution samples is usually necessary and should be done immediately if the filter is not positioned on the cannula.

General Chapters

Filtration also removes insoluble excipients that may otherwise interfere with the analytical finish. Selection of the proper filter material is important and should be accomplished, and experimentally justified, early in the development of the dissolution procedure. Important characteristics to consider when choosing a filter material are type, filter size, and pore size. The filter that is selected based on evaluation during the early stages of dissolution procedure development may need to be reconsidered at a later time point. Requalification has to be considered after a change in composition of the drug product or changes in the quality of the ingredients (e.g. particle size of microcrystalline cellulose).

Examples of filters used in dissolution testing can be cannula filters, filter disks or frits, filter tips, or syringe filters. The filter material has to be compatible with the media and the drug. Common pore sizes range from 0.20 to 70 µm, however, filters of other pore sizes can be used as needed. If the drug substance particle size is very small (e.g., micronized or nanoparticles), it can be challenging to find a filter pore size that excludes these small particles.

Adsorption of the drug(s) by the filter may occur and needs to be evaluated. Filter materials will interact with dissolution media to affect the recovery of the individual solutes and must be considered on a case-by-case basis. Different filter materials exhibit different drug-binding properties. Percentage of drug loss from the filtrate due to binding may be dependent on the drug concentration. Therefore the adsorptive interference should be evaluated on sample solutions at different concentrations bracketing the expected concentration range. Where the drug adsorption is saturable, discarding an initial volume of filtrate may allow the collection of a subsequent solution that approaches the original solution concentration. Alternative filter materials that minimize adsorptive interference can usually be found. Prewetting of the filter with the medium may be necessary. In addition, it is important that leachables from the filter do not interfere with the analytical procedure. This can be evaluated by analyzing the filtered dissolution medium and comparing it with the unfiltered medium.

The filter size should be based on the volume to be withdrawn and the amount of particles to be separated. Use of the correct filter dimensions will improve throughput and recovery, and also reduce clogging. Use of a large filter for small-volume filtration can lead to loss of sample through hold-up volume, whereas filtration through small filter sizes needs higher pressures and longer times, and the filters can clog quickly.

Filters used for USP Apparatus 4 need special attention because they are integrated in the flow-through process. Undissolved particles may deposit on the filters, creating resistance to the flow.

In the case of automated systems, selection of the filter with regard to material and pore size can be done in a similar manner to manual filtration. Flow rate through the filter and clogging may be critical for filters used in automated systems. Experimental verification that a filter is appropriate may be accomplished by comparing the responses for filtered and unfiltered standard and sample solutions. This is done by first preparing a suitable standard solution and a sample solution. For example, prepare a typical dissolution sample in a beaker and stir vigorously with a magnetic stirrer to dissolve the drug load completely. For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions.

1.2 Determining Solubility and Stability of Drug Substance in Various Media

Physical and chemical characteristics of the drug substance need to be determined as part of the process of selecting the proper dissolution medium. When deciding the composition of the medium for dissolution testing, it is important to evaluate the influence of buffers, pH, and if needed, different surfactants on the solubility and stability of the drug substance. Solubility of the drug substance is usually evaluated by determining the saturation concentration of the drug in different media at 37° using the shake-flask solubility method (equilibrium solubility). To level out potential ion effects between the drug and the buffers used in the media, mixtures of hydrochloric acid and sodium hydroxide are used to perform solubility investigations; this is in addition to the typical buffer solutions. In certain cases, it may be necessary to evaluate the solubility of the drug at temperatures other than 37° (i.e., 25°). The pH of the clear supernatant should be checked to determine whether the pH changes during the solubility test. Alternative approaches for solubility determination may also be used.

Typical media for dissolution may include the following (not listed in order of preference): diluted hydrochloric acid, buffers (phosphate or acetate) in the physiologic pH range of 1.2–7.5, simulated gastric or intestinal fluid (with or without enzymes), and water. For some drugs, incompatibility of the drug with certain buffers or salts may influence the choice of buffer. The molarity of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

Aqueous solutions (acidic or buffer solutions) may contain a percentage of a surfactant [e.g., sodium dodecyl sulfate (SDS), polysorbate, or lauryldimethylamine oxide] to enhance the solubility of the drug. The surfactants selected for the solubility investigations should cover all common surfactant types, i.e., anionic, nonionic, and cationic. When a suitable surfactant has been identified, different concentrations of that surfactant should be investigated to identify the lowest concentration needed to achieve sink conditions. Typically, the surfactant concentration is above its critical micellar concentration (CMC). *Table 1* shows a list of some of the surfactants used in dissolution media. Approximate CMC values are provided with references when available. The list is not comprehensive and is not intended to exclude surfactants that are not listed. Other substances, such as hydroxypropyl β-cyclodextrin, have been used as dissolution media additives to enhance dissolution of poorly soluble compounds. The U.S. Food and Drug Administration (FDA) maintains a database of dissolution methods, including information on dissolution media that have been used *(1)*. Typically, the amount of surfactant added is sufficient to achieve sink conditions in the desired volume of dissolution medium.

It is important to control the grade and purity of surfactants because use of different grades could affect the solubility of the drug. For example, SDS is available in both a technical grade and a high-purity grade. Obtaining polysorbate 80 from different sources can affect its suitability when performing high-performance liquid chromatography (HPLC) analysis.

There may be effects of counter-ions or pH on the solubility or solution stability of the surfactant solutions. For example, a precipitate forms when the potassium salt for the phosphate buffer is used at a concentration of 0.5 M in combination with SDS. This can be avoided by using the sodium phosphate salt when preparing media with SDS.

Table 1. Commonly Used Surfactants with Critical Micelle Concentrations

	Surfactant	CMC (% wt/volume)	Reference
	Sodium dodecyl sulfate (SDS), Sodium lauryl sulfate (SLS)	0.18%–0.23%	*(2–4)*
	Taurocholic acid sodium salt	0.2%	*(3)*
	Cholic acid sodium salt	0.16%	*(3)*
Anionic	Desoxycholic acid sodium salt	0.12%	*(3)*
	Cetyltrimethyl ammonium bromide (CTAB, Hexadecyltrimethylammonium bromide)	0.033%–0.036% (0.92–1.0 mM)	*(5,6)*
Cationic	Benzethonium chloride (Hyamine 1622)	0.18% (4 mM)	*(2)*
	Polysorbate 20 (Polyoxyethylene (20) sorbitan monolaurate, Tween 20)	0.07%–0.09%	*(3,7)*
	Polysorbate 80 (Polyoxyethylene (20) sorbitan monooleate, Tween 80)	0.02%–0.08%	*(3,7)*
	Caprylocaproyl polyoxyl-8 glycerides (Labrasol)	0.01%	*(4)*
	Polyoxyl 35 castor oil (Cremophor EL)	0.02%	*(8)*
	Polyoxyethylene 23 lauryl ether (Brij 35)	0.013%	*(9)*
Nonionic	Octoxinol (Triton X-100)	0.01%–0.03%	*(3,10)*
Zwitterion	Lauryldimethylamine *N*-oxide (LDAO)	0.023%	*(11)*

Routinely, the dissolution medium is buffered; however, the use of purified water as the dissolution medium is suitable for products with a dissolution behavior independent of the pH of the medium. There are several reasons why purified water may not be preferred. The water quality can vary depending on its source, and the pH of the water is not as strictly controlled as the pH of buffer solutions. Additionally, the pH can vary from day to day and can also change during the run, depending on the drug substance and excipients. Use of an aqueous–organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

Investigations of the stability of the drug substance should be carried out, when needed, in the selected dissolution medium with excipients present, at 37°. This elevated temperature has the potential to decrease solution stability (degradation). Stability should allow for sufficient time to complete or repeat the analytical procedure. Physical stability may be of concern when precipitation occurs because of lower solubility at room or refrigerated temperature.

1.3 Choosing a Medium and Volume

When developing a dissolution procedure, one goal is to have sink conditions, which are defined as having a volume of medium at least three times the volume required to form a saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide sink conditions may be acceptable if it is appropriately justified. The composition and volume of dissolution medium are guided by the solubility investigations. For example, the choice and concentration of a surfactant need to be justified from the solubility data and the dissolution profiles.

The use of enzymes in the dissolution medium is permitted, in accordance with *Dissolution* ⟨711⟩, when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products. A discussion of the phenomenon of cross-linking and method development using enzymes can be found in *Capsules–Dissolution Testing and Related Quality Attributes* ⟨1094⟩. Validation should be performed with the method using enzymes according to section *5. Validation*.

Another option is to use media that follow more closely the composition of fluids in the stomach and intestinal tract. These media may contain physiological surface-active ingredients, such as taurocholates. The media also may contain emulsifiers (lecithin) and components such as saline solution that increase osmolality. Also, the ionic strength or molarity of the buffer solutions may be manipulated. The media are designed to represent the fed and fasted state in the stomach and small intestine. These media may be very useful in modeling in vivo dissolution behavior of immediate-release (IR) dosage forms, in particular those containing lipophilic drug substances, and may help in understanding the dissolution kinetics of the product related to the physiological make-up of the digestive fluids. Results of successful modeling of dissolution kinetics have been published, mainly for IR products. In the case of extended-release dosage forms with reduced effect of the drug substance on dissolution behavior, the use of such media needs to be evaluated differently. In vitro performance testing does not necessarily require media modeling the fasted and postprandial states *(12,13)*.

An acid stage is part of the testing of delayed-release products by *Method A* or *Method B* in ⟨711⟩. For drugs with acid solubility less than 10% of the label claim or drugs that degrade in acid the usefulness of the acid stage in detecting a coating failure is compromised. This would be handled on a case-by-case basis. Possible resolutions include the addition of surfactant to the acid stage, or adjustment of the specifications.

During selection of the dissolution medium, care should be taken to ensure that the drug substance is suitably stable throughout the analysis. In some cases, antioxidants such as ascorbic acid may be used in the dissolution medium to stabilize the drug. There are occasions where such actions are not sufficient. For compounds that rapidly degrade to form a stable degradant, monitoring the degradant alone or in combination with a drug substance may be more suitable than analyzing only the drug substance. In situ spectroscopic techniques tend to be less affected by degradation when compared with HPLC analysis (including UHPLC and other liquid chromatographic approaches).

For compendial Apparatus 1 (basket) and Apparatus 2 (paddle), the volume of the dissolution medium can vary from 500 to 1000 mL. Usually, the volume needed for the dissolution test can be determined in order to maintain sink conditions. In some cases, the volume can be increased to between 2 and 4 L, using larger vessels and depending on the concentration and sink conditions of the drug; justification for this approach is expected. In practice, the volume of the dissolution medium is usually maintained within the compendial range given above. Alternatively, it may be preferable to switch to other compendial apparatus, such as a reciprocating cylinder (Apparatus 3), reciprocating holder (Apparatus 7), or flow-through cell (Apparatus 4). Certain applications may require low volumes of dissolution media (e.g., 100–200 mL) when the use of a paddle or basket is preferred. In these cases, an alternative, noncompendial apparatus (e.g., small-volume apparatus) may be used.

1.4 Choosing an Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. In general, a compendial apparatus should be selected.

For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. When Apparatus 1 or Apparatus 2 is not appropriate, another official apparatus may be used. Apparatus 3 (reciprocating cylinder) has been found especially useful for chewable tablets, soft gelatin capsules, delayed-release dosage forms, and nondisintegrating-type products, such as coated beads. Apparatus 4 (flow-through cell) may offer advantages for modified-release dosage forms and immediate-release dosage forms that contain active ingredients with limited solubility. In addition, Apparatus 4 may have utility for multiple dosage form types such as soft gelatin capsules, beaded products, suppositories, or depot dosage forms, as well as suspension-type extended-release dosage forms. Apparatus 5 (paddle over disk) and Apparatus 6 (rotating cylinder) are useful for evaluating and testing transdermal dosage forms. Apparatus 7 (reciprocating holder) has application to non-disintegrating, oral modified-release dosage forms, stents, and implants, as well as transdermal dosage forms. For semisolid dosage forms, the generally used apparatus include the vertical diffusion cell, immersion cell, and flow-through cell apparatus with the insert for topical dosage forms (see *Semisolid Drug Products—Performance Tests* ⟨1724⟩).

Some changes can be made to the compendial apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10-, 20-, or 80-mesh) may be used when the need is clearly documented by supporting data. Care must be taken that baskets are uniform and meet the dimensional requirements specified in ⟨711⟩.

A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. A rotating bottle or dialysis tubes may have utility for microspheres and implants, peak vessels, and modified flow-through cells for special dosage forms including powders and stents.

2. METHOD DEVELOPMENT

A properly designed test should yield data that are not highly variable, and should be free of significant stability problems. High variability in the results can make it difficult to identify trends or effects of formulation changes. Sample size can affect the observed variability. One guidance defines dissolution results as highly variable if the relative standard deviation (RSD) is more than 20% at time points of 10 min or less and more than 10% at later time points for a sample size of 12 *(14)*. However, during method development, smaller sample sizes may be used, and the analyst will need to make a judgment accordingly. Most dissolution results, however, exhibit less variability. In the development of a dissolution procedure the source of the variability should be investigated, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing any of the following factors: the apparatus type, speed of agitation, level of deaeration, sinker type, or composition of the medium.

Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, excipient interactions or interference, film coating, capsule shell aging, and hardening or softening of the dosage form on stability may be sources of variability and interferences.

2.1 Deaeration

The significance of deaeration of the dissolution medium should be determined because air bubbles can act as a barrier to the dissolution process if present on the dosage unit or basket mesh and can adversely affect the reliability of the test results. Furthermore, bubbles can cause particles to cling to the apparatus and vessel walls. Bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. Poorly soluble drugs are most sensitive to interference from air bubbles; therefore, deaeration may be needed when testing these types of products. A deaeration method is described as a footnote in the *Procedure* section of ⟨711⟩. Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deaeration are available and are in routine use throughout the industry. Once a suitable deaeration process is identified, it should be documented as part of the dissolution procedure. The extent of deaeration can be evaluated by measuring the total dissolved gas pressure or by measuring the concentration of dissolved oxygen in water. For example, an oxygen concentration below 6 mg/L has been found effective as a marker for adequate deaeration of water for the Performance Verification Test with USP Prednisone Tablets RS.

Media containing surfactants usually are not deaerated because the process results in excessive foaming, and usually the effect of dissolved air on the dissolution process is mitigated by the reduced surface tension of the medium. Sometimes, deaerating the medium before adding surfactants can be effective.

To determine whether deaeration of the medium is necessary, compare results from dissolution samples run in non-deaerated medium and medium deaerated using a compendial technique, as described above. If no effect of deaeration is detected, this experiment could serve as justification that deaeration is not required in the future. If there is an effect, however, then it is necessary to carefully control this parameter, and it is prudent to characterize the robustness of the deaeration process. The dissolved gas content of deaerated media under atmospheric pressure is unstable and will tend toward saturation. Manipulations of the deaerated medium such as stirring or pouring can increase the rate at which atmospheric gases are redissolved.

2.2 Sinkers

Sinkers are often used to adjust the buoyancy of dosage forms that would otherwise float during testing with Apparatus 2. When sinkers are used, a detailed description of the sinker must be provided in the written procedure. It may be useful to evaluate different sinker types, recognizing that sinkers can significantly influence the dissolution profile of a dosage unit. When transferring the procedure, the same sinkers should be used, or if a different design is used, it should be shown to produce equivalent results. There are several types of commercially available sinkers. In ⟨711⟩, a harmonized sinker is described in *Figure 2a*.

A standard sinker can be made by using the appropriate length of wire and coiling it around a cylinder. For materials, use 316 stainless steel wire, typically 0.032 inch/20 gauge, or other inert material and wind the wire around cylinders of appropriate diameter (e.g., cork borers) for an appropriate number of turns to fit the capsule shell type. Sizes are shown in *Table 2*. The ends of the coil can be curved to retain the capsule within the sinker when they are immersed. Because the ends of the wire may be rough, they may need to be filed. If the sinker is handmade, the sinker material and construction procedure instructions should be documented (e.g., dimension, design, number of coils); if a commercial sinker is used, the vendor part number should be reported if available.

Table 2. Wire Sinkers Used With Common Capsule Shell Sizes

Capsule Shell Size	Length of Wire (cm)	Diameter Size (cm)	Cork Bore Number
#0, elongated	12	0.8	4
#1 and #2	10	0.7	3
#3 and #4	8	0.55	2

Although sinkers are typically used to keep the dosage form at the bottom of the vessel, they can also be used to keep dosage forms from sticking to the vessel (e.g., film-coated tablets). The sinker should be appropriate to the dosage form; therefore, the same sinker size may not be suitable for all dosage-form sizes. The sinker should not be too tight around the dosage form because this may restrict interaction with the medium. Conversely, if wrapped too loosely, the dosage form may escape soon after the test begins. The sinker should be small enough that the capsule does not change its orientation within the sinker. Care should be taken when testing capsules that have some cross-linking present, to keep the sticky shell from attaching to the vessel bottom. In this case, the harmonized sinker design provided in *Figure 2a* of ⟨711⟩ will be advantageous.

2.3 Agitation

For immediate-release capsule or tablet formulations, Apparatus 1 (baskets) at 50–100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are used commonly. Other agitation speeds are acceptable with appropriate justification. Rates outside 25–150 rpm for both the paddle and the basket are usually not appropriate because of mixing inconsistencies that can be generated by stirring too slow or too fast. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions.

For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and improving the data. If justified, 100 rpm may be used with Apparatus 2, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if to achieve an in-vitro–in-vivo correlation (IVIVC) the resulting profiles better reflect in vivo performance, or if the method results in better discrimination without adversely affecting method variability.

Apparatus 3 (reciprocating cylinder) can be used at dip rates ranging from 5 to 30 dips/min. The hydrodynamics are influenced by the cylinder's reciprocating motion and the resulting movement of the sample in the medium. The reciprocating motion of the cylinder and screen may cause foaming if the medium contains surfactants. Addition of an anti-foaming agent such as simethicone or *n*-octanol may be useful for avoiding foaming from surfactants.

Apparatus 4 (flow-through cell) is described in ⟨711⟩ with standard flow rates of 4, 8, and 16 mL/min. Other flow rates for Apparatus 4 can be used if justified and if within the capacity of the pump to conform with the requirements in ⟨711⟩. Agitation in Apparatus 4 is not only related to the pump speed but can also be affected by cell diameter. At a set flow rate, as measured by volume, the 12-mm cell will develop a greater linear fluid velocity than is achieved in the 22.6-mm cell. Apparatus 4 can be configured with the addition of glass beads in the entry cone of the flow-through cell (packed column) or without glass beads (open column).

The flow characteristics of the flow-through cell are discussed in the scientific literature (*15*). The placement of the sample in the flow-through cell will influence the flow patterns that occur and thus should be a consideration in the attempt to reduce variability of the results.

2.4 Study Design

Selection of the agitation rate and other study design elements for the dosage form, whether immediate release or modified release, should conform to the requirements and specifications (i.e., apparatus, procedures, and interpretation) given in ⟨711⟩.

2.4.1 TIME POINTS

For immediate-release dosage forms, the duration of the dissolution procedure is typically 30–60 min; in most cases, a single time point specification is adequate for pharmacopeial purposes. For method development, however, a sufficient number of time points should be selected to adequately characterize the ascending and plateau phases of the dissolution curve. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. According to the Biopharmaceutics Classification System referred to in several FDA Guidances, highly soluble, highly permeable drugs formulated into very rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the drug substance within 15 min. For these types of products, a one-point test or disintegration will suffice. However, most products do not fall into this category. Dissolution profiles of immediate-release products typically show a gradual increase reaching 85%–100% at about 30–45 min. Thus, sufficient dissolution time points are chosen to characterize the performance for most immediate-release products. For some products, including suspensions, useful information may be obtained from earlier points, e.g., 5–10 min. For slower-dissolving products, time points later than 60 min may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

The f_2 similarity factor may not be useful when more than 85% is dissolved at 15 min. If the f_2 similarity factor is to be used, multiple time points for the dissolution test are required, with at least two time points with mean percent dissolved (typically for $n = 12$) below 85% dissolved and only one point above 85% for both products (*16*). Therefore, the addition of early time points may be useful.

For testing an extended-release dosage form, at least three time points are chosen, to guard against dose dumping, to define the in vitro release profile, and to show that essentially complete release (>80%) of the drug is achieved. Additional sampling times may be useful. Certain IVIVC criteria, such as level B correlation (according to *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩), require the experimental determination of the time to dissolve 100% of the label claim. Selection of the final time points is reflective of the data from the drug release profile that are generated during development. For products containing more than a single active ingredient, determine the drug release for each active ingredient.

Delayed-release dosage forms usually require specifications for at least two time points; therefore, it is important during development to evaluate the entire dissolution profile. In the case of enteric-coated dosage forms, the functionality of the coating is usually proven by challenge in an acid medium, followed by a demonstration of dissolution in a higher-pH medium. Chapter ⟨711⟩ gives a standard buffer medium for that stage of testing but other media may be used if justified. The timing of the acid stage is typically 2 h, and release in the buffer is similar to the timing for immediate-release forms. For delayed-release dosage forms that are not enteric coated, setting of specifications is different. Unlike delayed release, the onset of release is not determined by the experimental design, which is the pH change; multivariate specifications, therefore, may be needed to define time ranges and corresponding percentage ranges.

So-called infinity points can be useful during development studies. To obtain an infinity point, the paddle or basket speed is increased at the end of the run (after the last time point) for a sustained period (typically, 15–60 min), after which time an additional sample is taken. Although there is no requirement for 100% dissolution in the profile, the infinity point can be com-

pared to content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

2.4.2 OBSERVATIONS

Visual observations and recordings of product dissolution and disintegration behavior are useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. For visual observation, proper lighting (with appropriate consideration of photo-degradation) of the vessel contents and clear visibility in the bath are essential. Documenting observations by drawing sketches and taking photographs or videos can be instructive and helpful for those who are not able to observe the real-time dissolution test. Observations are especially useful during method development and formulation optimization. It is important to record observations of all six vessels to determine if the observation is seen in all six vessels, or just a few. If the test is performed to assist with formulation development, provide any unique observations to the formulator. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus (e.g., below the basket or paddle), when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit, especially at the surface of the media.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products, [e.g., the partial opening and splitting apart (similar to a clamshell) or incomplete opening of the shell], accompanied by the release of air bubbles and excipients.
9. Whether the dosage form lands in the vessel center or off-center, and if off-center, whether it sticks there.
10. Time required for the complete dissolution of the capsule shell or for tablet disintegration.

Observations also help to document that the proper procedure has been followed, or more importantly, that a deviation has occurred. Examples include the confirmation that a dosage form is actually in the vessel during the test or that more than one dosage form are inadvertently in the same vessel, or that a filter from the autosampler has dropped into the vessel.

2.4.3 SAMPLING

Manual: For manual sampling, use chemically inert devices (e.g., polymeric or glass syringes, and polymeric or stainless steel cannula), a filter, and/or a filter holder. The sampling site must conform to specifications in ⟨711⟩. When the agitation conditions are very slow, e.g., a 50-rpm basket, care should be taken to sample consistently in the same location in the vessel because there may be a concentration gradient; avoid sampling very close to the shaft or vessel wall. During method development, a decision should be made regarding whether to replace the media after each time point. Replacement is not preferred because the dosage unit may be disturbed during delivery of the media. However, replacement may be necessary if maintaining sink conditions is a challenge. With replacement, the volume used in the calculations remains the same throughout the time points, but there is some drug substance withdrawn with each sample that will need to be accounted for in the calculations.

Metal surfaces may interact with the sample. For example, adsorption onto metal surfaces may occur, or the metal surfaces may release metal ions into aqueous media. The ions can then catalyze degradation reactions, leading to artifacts during the analytical procedures. The surfaces of stirring elements and metal locks of syringes may be sources of interference to accurate sampling.

Autosampling: Autosampling is discussed in section *4. Automation*.

2.4.4 CLEANING

Importance is placed on evaluation of the cleaning process between tests. Changes of dissolution medium and/or product necessitate the need for cleaning. Residues on the vessels can affect the results (e.g., adsorbed residues may dissolve and alter subsequent media properties or interfere with the sample analysis), and effective cleaning will return them to a suitable state. Automated systems are discussed in section *4.4 Cleaning*.

General Chapters

2.5 Data Handling

Dissolution rates are calculated from the change in drug concentration in the dissolution medium. For procedures in which the volume of medium is fixed, such as for Apparatus 1 and Apparatus 2 testing of immediate-release dosage forms with only one sampling time, the concentration of the sample is multiplied by the medium volume to arrive at the mass of drug dissolved usually expressed as percentage of label claim. When multiple time points are taken, the total amount of drug removed at earlier time points should be assessed and may be part of the calculation of the amount dissolved, if considered important. Similarly, if the medium volume is not fixed, for example when the sample volume is not replaced in testing extended-release products, the change in medium volume must be part of the calculation for successive sampling points. Dissolution tests performed with Apparatus 4 in the closed-loop configuration with in situ detection provide a convenient control of the medium volume. For testing with Apparatus 4 in the open configuration, the test time and flow rate will determine the volume of medium used in the dissolution calculations.

Dissolution results can be evaluated as either cumulative rates or fractional rates. Cumulative rates represent the sum of all drug dissolution that occurs during an interval (*Figure 1*). Fractional rates are assessed at a specific time point or during a portion of the total test time (*Figure 2*). Typically, the rate of release will be expressed as either mass or percentage of label claim per unit time. For most compendial dissolution testing, the dissolution rate is expressed as a percentage of the label claim dissolved at the indicated test time.

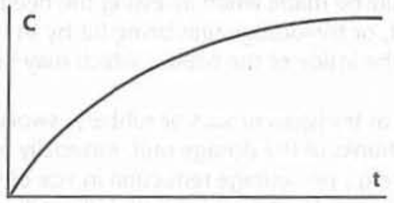


Figure 1. An example of a plot of dissolution as a cumulative process. Concentration, C, is the amount of drug released per volume of medium, and t represents time. This type of plot is readily observed in constant-volume dissolution systems, such as Apparatus 1 or Apparatus 2, or Apparatus 4 in closed-loop configuration.

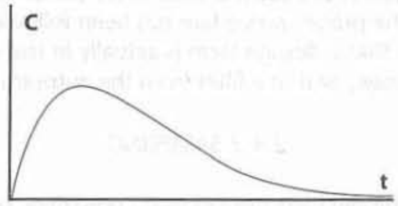


Figure 2. An example of a plot of the observed concentration of the sample taken for an interval that is negligibly small in relation to the time of the overall dissolution process. This concentration is propostional to the instantaneous or fractional dissolution rate (*dc*/*dt*). This type of plot is readily observed in continuous-flow dissolution systems, such as Apparatus 4 in open-loop configuration.

Cumulative dissolution profiles represent the total amount of drug dissolved from the formulation over time. When cumulative dissolution is measured in a constant-volume system, no correction for the amount lost in sampling needs to be made. If sample is removed from the system, the amount consumed in analysis must be accounted for in the calculation. Recirculated sampling with Apparatus 1 or Apparatus 2, or with Apparatus 4 in the closed-loop configuration (*Figure 3*), are all examples of systems that will produce cumulative dissolution rates. With Apparatus 4 in the open configuration (*Figure 4*), cumulative rates accounting for the total amount of drug dissolved across the testing interval are obtained by collecting and analyzing the entire outflow from each individual flow-through cell. With Apparatus 3 (*Figure 5*), the medium in each tube is sampled at the end of the programmed interval, and the analyzed concentration represents the cumulative dissolution rate during that interval.

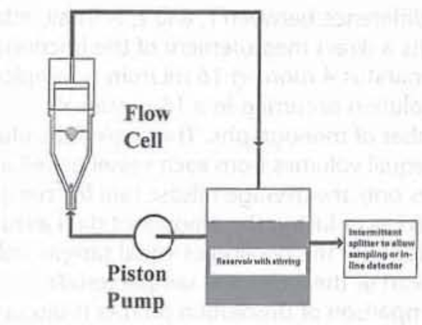


Figure 3. Apparatus 4 in the closed-loop configuration.

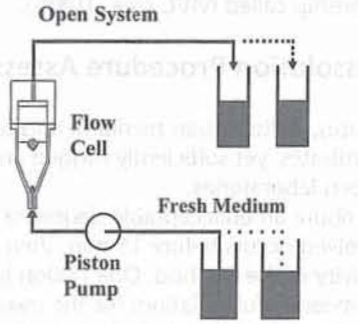


Figure 4. Apparatus 4 in the open-loop configuration. The sample can be collected in fractions, as shown at the top. The medium can be changed by using successive reservoirs.

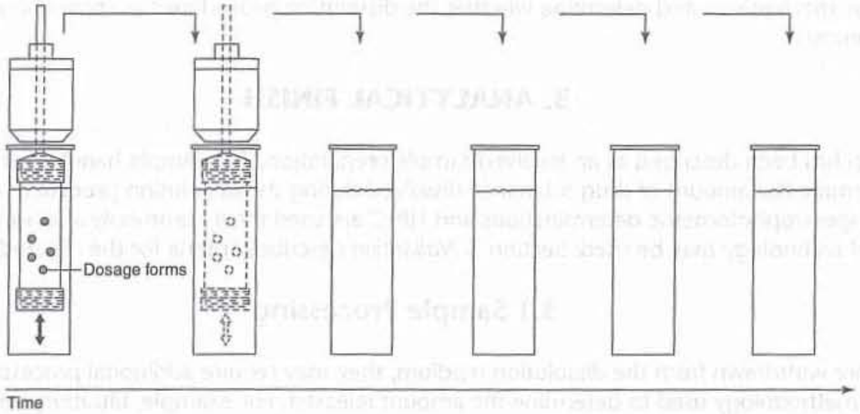


Figure 5. The progression that is possible for one reciprocating cylinder from Apparatus 3. The reciprocating cylinder can move from vessel to vessel. This feature facilitates changing the dissolution medium and testing for different intervals in successive tubes.

Fractional dissolution rates are typically measured for a discrete interval. A series of such rates will produce a step function as the dissolution profile. At any time, the cumulative dissolution rate from this type of profile is the sum of the preceding intervals. This type of profile is represented by Apparatus 3 using multiple tubes or Apparatus 4 in the open-loop configuration where the total outflow is collected and analyzed for successive intervals.

A number of algebraic and numerical methods exist for transforming cumulative and fractional dissolution results. The difference in amount released for successive time points can be calculated, and the average release rate is determined by the formula:

$$\text{Result} = (M_2 - M_1)/(t_2 - t_1)$$

M = mass or percentage of label claim
t = time

As the difference of t_2 from t_1 is reduced, the average rate can be considered to approach an instantaneous rate. Sampling considerations and physical constraints on measurement of the mass transfer at the medium interface of the dosage form make the measurement of true instantaneous dissolution impractical for routine determination in the laboratory. Fractional dis-

solution is measured for intervals where the difference between t_2 and t_1 is small, relative to the total test time. The design of Apparatus 4 in the open configuration permits a direct measurement of the fractional dissolution over small time intervals. For example, if a 4-mL fraction of outflow for Apparatus 4 running 16 mL/min is sampled, either by in situ detection or offline, the amount of drug detected represents the dissolution occurring in a 15-s interval.

Pooled dissolution has been used in a number of monographs. The pooled dissolution procedure produces an average release rate for the units tested by combining equal volumes from each vessel or cell and performing analysis of only the one resulting solution. Because this approach uses only the average release rate for comparison with the acceptance table, the pooled dissolution procedure has been viewed as reducing the amount of data available from the dissolution test and, thus, reducing its value. However, it should be noted that the pooling of equal sample volumes is equivalent, from a calculation standpoint, to determining the arithmetic mean of the individual sample results.

The use of the f_2 similarity factor in the comparison of dissolution profiles is discussed in *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* 〈1090〉.

For the purpose of correlation with in vivo data, parameters of mathematical models are obtained by fitting to dissolution data to establish a continuous functional relationship called IVIVC (see 〈1088〉).

2.6 Dissolution Procedure Assessment

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that together provide a method that is sensitive to changes in critical quality attributes, yet sufficiently rugged and reproducible for day-to-day operation. The method should be able to be transferred between laboratories.

The ideal dissolution procedure will not contribute an unacceptable degree of variability and will provide a profile with adequate points below 85% dissolved. If 85% dissolved occurs before 15 min, then f_2 comparisons may not be appropriate.

There are many ways to challenge the sensitivity of the method. One option is to compare dissolution profiles of formulations that are intentionally manufactured with meaningful variations for the most relevant critical manufacturing variable, for example, ±10%–20% change to the ranges of these variables. Similarly, samples that have been stressed may be used to demonstrate sensitivity to changes on stability. This concept may be used to establish the factors that are most significant in their influence on the dissolution rate. These studies can focus on either the dissolution parameters (e.g., media concentration, agitation rate, and deaeration) or the product attributes (e.g., excipient ratios, particle size, compression). The ultimate goal is to understand the release mechanisms and determine whether the dissolution procedure can show change in the critical quality attributes of a drug product.

3. ANALYTICAL FINISH

The dissolution step has been described as an involved sample preparation. The sample handling and analytical procedure that are used to determine the amount of drug substance dissolved during the dissolution procedure are termed the "analytical finish." Although spectrophotometric determinations and HPLC are used most commonly and are discussed in this chapter, any suitable analytical technology may be used. Section *5. Validation* describes criteria for the methods.

3.1 Sample Processing

After the samples are withdrawn from the dissolution medium, they may require additional processing to make them suitable for the analytical methodology used to determine the amount released. For example, filtration may be used to remove undissolved particulate matter, or samples may need to be protected from exposure to light or may need refrigerated storage. In addition, samples may have to be diluted to a level that is within the linear range of the method. With analysis by HPLC, dilution of the sample with mobile phase may be necessary to reduce the effect on the separation of injecting dissolution medium. Other types of treatment may be necessary depending on the product formulation, such as the inactivation or elimination of interference caused by components of the formulation by the addition of appropriate reagents. However, separation may not be possible or needed in all cases. In some cases, in situ measurements obtained with methods such as fiber optics or electrochemical determination may be useful.

3.2 Filters

The topic of filtration is discussed in section *1.1 Performing Filter Compatibility*.

3.3 Centrifugation

Centrifugation of samples is not preferred, for several reasons: dissolution can continue to occur until the solids are removed, a concentration gradient may form in the supernatant, and energy imparted may lead to increased dissolution of the drug substance particles. Possible exceptions, when centrifugation could be preferred, might include the use with compounds that adsorb onto all common filters, or situations when the potential filter leachables and extractables might interfere in the quanti-

tative step of the dissolution test (e.g., when fluorescence procedures are used in quantitation). Centrifugation may prove useful during method development for evaluating the suitability of the filter material.

3.4 Analytical Procedure

The usual assay for a dissolution sample employs either a spectrophotometric procedure or a liquid chromatographic procedure. Spectrophotometric determination may be direct or may provide the detection for HPLC. Spectrophotometric determination is used often because results can be obtained faster, the analysis is simpler, it is easier to automate, and fewer solvents are needed. The use of direct spectrophotometric determination typically requires confirmation of specificity. HPLC is preferred for a number of reasons such as providing a wide dynamic range that reduces the need to dilute some samples while also providing sensitivity in the analysis of dilute samples, and greater selectivity when excipients or multiple drugs in the formulation present a significant interference. Modern HPLC systems employ autosamplers that provide speed and simplicity advantages comparable to spectrophotometric analysis.

3.5 Spectrophotometric Analysis

Direct spectrophotometric analysis may be performed on samples that are manually introduced to the cuvette. Alternatively, samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance, as described in the standard operating procedures or metrology documents, help to ensure reliable operation of these instruments. Cells with path lengths ranging from 0.02 cm to 1 cm are typically used, and longer path-length cuvettes can be used to increase the range for quantification of dilute samples. Cell alignment and air bubbles could be sources of error. The shorter path-length cells are used to avoid diluting the sample; in all cases, however, acceptable linearity and standard error need to be demonstrated.

The choice of wavelength for the determination should be based on the spectrum of the drug in solution. In some cases, where the drug substance can degrade in the dissolution medium (e.g., dosage forms containing aspirin), it is useful to carry out the measurements at the isosbestic point. Excipients can also have effects, but performing analysis at multiple wavelengths can minimize their effects. The contribution of the absorbance from an excipient at the analytical wavelength can sometimes be determined by ratio from its absorbance at a wavelength where the absorbance of the drug substance is minimal.

Using a validated analytical finish, standard solutions are typically prepared in dissolution media and analyzed at just one concentration, either at 100% of the dosage strength or the selected *Q* value because linearity of the analytical finish has been established. Prior to validation, dissolution profile analysis, or analysis of products of various strengths, requires using multiple standard solutions covering the expected range of concentration. A typical media blank, standard, and sample may be analyzed in a sequence that brackets the sample with standards and blanks, especially at the beginning and end of the analysis. The standard and sample solutions should both be prepared in the dissolution medium in the linear concentration range and measured at the same wavelength. However, small amounts of an organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg/mL, divided by the cell path length in cm. A rearrangement of the Beer-Lambert expression gives the absorptivity, a, as:

$$a = A/bc$$

A = absorbance
b = path length (cm)
c = concentration (mg/mL)

Typical units for absorptivity that are used for dissolution testing are in terms of AU · mL/mg, where AU is absorbance unit. Historical data may be used to provide an acceptable absorptivity range for the analyte (using the appropriate path-length cell). This value may be useful in troubleshooting aberrant data.

Fiber optics as a sampling and determinative method, with proper validation, are an option.

3.6 HPLC

For HPLC analysis, the effect on the chromatogram of peaks resulting from injection of dissolution media require enumeration. A large solvent disturbance may affect accuracy and precision of response if it is poorly resolved from the peak of interest. This is even more important if large injector volumes (>100 μL) are needed. System suitability tests may evaluate peak shape; separation of the main peak from solvent disturbance and from closely eluting peaks; and injection precision. At a minimum, the precision is critical.

Ideally, the standard solutions should be diluted with the dissolution media at a concentration within the linear range of the method, e.g., 100%, or the selected *Q* value of the dosage strength. However, organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation. In some cases, the sample may be diluted with mobile phase to improve the peak shape. The standard and sample solutions should both be prepared in the linear concentration range and measured at the same wavelength.

4. AUTOMATION

Automated dissolution systems may be configured in various ways and degrees. The elements of test preparation, initiation, sampling and timing, and cleaning all can be automated. Fully automated systems are available, as are systems where individual steps, such as media preparation or sampling, are automated. This section will discuss operational steps that can be automated. The level of complexity for automation depends on whether the instrument configuration is open or closed loop and also whether the analytical device is coupled online or offline. Online analysis returns the sample aliquot to the test system, as in the case of spectrophotometry with flow-through cuvettes. Offline analysis removes the sample aliquot from the dissolution medium for subsequent analysis, typically by HPLC, where the analysis consumes the sample. The decision on the configuration usually depends on the number of samples to be processed and the time required for their analysis.

Automation may require deviations from the pharmacopeial specifications of the instruments, such as incorporation of an integrated outlet on the bottom of the vessel for cleaning and replacement of medium.

Operational steps that are not part of the compendial procedure should be validated. Deviations from the standard procedure described in ⟨711⟩, such as use of sampling probes or fiber-optic probes, should be validated against the standard procedure.

4.1 Medium Preparation

Automated media preparation generally is accomplished by diluting concentrates. Automated media preparation systems typically dispense the volume of medium into the vessel by monitoring either the weight or volume. Chemical and physical stability of the concentrates as well as homogeneity of the dilutions over the intended period of use are important issues and should be understood. Concentrates of buffer solutions and surfactants may have stability issues, such as chemical degradation and pH change. Physical instability may manifest as precipitation, re-crystallization, or phase separation and should be prevented.

If deaeration of the medium is required, the level of deaeration should be specified.

The concentration of the dissolved oxygen can be used to evaluate the efficiency of deaeration procedures discussed in section *2.1 Deaeration*.

4.2 Sample Introduction and Timing

Samples should be inserted in the vessel in a reproducible way. Automated sample introduction and aliquot withdrawal provide an advantage over manual sampling because the automated techniques can reduce the variability in the vessel-to-vessel timing of the test intervals. However, automated sample handling may impose timing limitations that need to be considered. The pharmacopeial tolerance of ±2% of the specified dissolution test time may be difficult to meet for early time points.

4.3 Sampling and Filtration

Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. The transfer and filtration of sample solutions from the dissolution instrument to the analytical unit may be undertaken via tube connections or via robotic devices operated in a stepwise procedure. Sample volumes may be removed from the dissolution medium and not returned (consumptive sampling), or the sample volume may be returned to the dissolution medium (recirculated sampling).

There are many brands of autosamplers, including semi-automated and fully automated systems. Routine performance checks, cleaning, and maintenance, as described in the pertinent standard operating procedures or metrology documents, help to ensure reliable operation of these devices.

Sampling probes may or may not remain in the vessel throughout the entire run. Sampling probes or fiber-optic probes can disturb the hydrodynamics of the vessel; therefore, adequate validation should be performed to ensure that the probes are not causing a significant change in the dissolution rate. If filters are used that are different from those used for manual sampling, then these different filters should also be evaluated separately. The position of the pharmacopeial sampling zone for Apparatus 1 and Apparatus 2 is midway from the top of the stirring element to the medium surface and depends on the medium volume. Sampling probes should pull the sample from the sampling zone. Instruments for which the sampling occurs through the hollow shaft should be designed with a means to adjust the depth of the inlet aperture to allow conformance with this requirement. The programmed sampling volume depends on the dead volume of the tubing, cuvettes, and other devices and has to be adjusted accordingly.

A recirculated sampling alignment can be operated either by discharging the tubing contents into the vessel after each sampling or by allowing the tubing to remain filled with solution in the intervals between sampling points. In the latter case, the dead volume and carryover effects are important considerations.

The need for sample volume replacement should be considered. In consumptive sampling with multiple sampling time points, the withdrawn volume may be replaced with an equal volume of fresh medium. The sampling volume may be critical if, in total, it exceeds 1% of the stated volume of dissolution medium required by the procedure. If it can be shown that re-

placement of the medium is not necessary, the volume change must be part of the calculation of results. See section *2.5 Data Handling*.

Carryover may occur when subsequent samples are affected by residues or conditions of previous samples; the effect of the first sample or condition "carries over" to the second. In liquid handling, residues of liquids previously in the sample solution may contaminate subsequent sample solutions. Dissolution media containing surfactants or lipids may present problems. Carryover may occur for successive samples taken over a multiple time-point test, as well as at the beginning of a new test due to the cleaning solution. This topic is discussed in section *4.4 Cleaning*.

Interaction of dissolved drug substance with the sampling and transfer devices is an important consideration. When adsorption of the dissolved drug substance occurs, it most often involves surfaces of the dissolution apparatus or sampling filters and tubing. Adsorption may be pH dependent in the case of charged, dissolved drug substance. Adsorption of the dissolved drug to the parts of the sampling device should be assessed using a typical sample solution (dissolution sample from the product or drug substance with formulation matrix) with known concentration. The typical design is a cross-validation with aliquots of the same sample solution passing and bypassing the sampling device (including the sampling probe, filter, tubing, valves, and pump). There is no general recommendation that may give preference to any kind of material or equipment construction (e.g., glass or specific polymers). See section *5.7 Considerations for Automation* for more information.

In addition to the information in section *2.4.3 Sampling*, connections of pumps and tubing may be sources of contamination in automated systems. Interferences with the spectroscopic analytical procedures, which are commonly used for dissolution testing, are less of a concern. However, interferences must be evaluated if the product under investigation contains low-dose metal salts, as do some dietary supplements.

Liquid transfer usually is undertaken via polymeric tubing. Inert materials such as polytetrafluoroethylene (PTFE) sometimes cannot be used because of their mechanical properties. Where flexible tubes are required, for example in peristaltic pumps or for coiling in a small radius, polypropylene (PP) or high-density polyethylene (HDPE) may be the preferred materials. Depending on the type of polymer and its crystallinity and density, leaching of constituents, mainly plasticizers, may occur. Leachables can interfere with the analytical procedure. The concentration leached to the sample solution usually depends on the surface, the temperature, the exposure time, the hydrodynamic conditions, and the composition of the media.

4.4 Cleaning

In addition to the information in section *2.4.4 Cleaning*, automated systems have specific cleaning issues. For example, evaluation of the effectiveness of purging and rinsing between sampling times and within-run condition of the tubing is recommended. Also it is important to evaluate the cleaning process between tests.

4.5 Operating Software and Computation of Results

The software systems for data evaluation and instrument operation must be validated as per 21 CFR 11 *(17)*.

4.6 Common Deviations from the Compendial Procedures That May Require Validation

Some common areas of deviation from compendial procedures include the following:

- Sample introduction relative to start of spindle rotation
- Residence time and positioning of sampling probes
- Recirculated versus consumptive sampling
- Sample volume replacement in consumptive sampling.

5. VALIDATION

The validation topics described in this section are typical but not all-inclusive and can be viewed in the context of *Validation of Compendial Procedures* 〈1225〉, as well as the International Conference on Harmonization (ICH) document, *Validation of Analytical Procedures (18)*. Validation for both parts of the dissolution procedure, the analytical finish and the dissolution step, will be discussed in this section. The dissolution step is the release of the drug in the dissolution medium and sampling. The analytical finish is defined in section *3. Analytical Finish*. Validation of the analytical finish will evaluate the attributes, linearity and range, precision, specificity, accuracy/recovery, robustness, and stability of the sample and standard solutions. Validation of the dissolution step will include evaluation of precision and robustness of the dissolution sample preparation. Validation of the analytical finish is performed either using a standard solution or spiked placebo or by the method of standard addition (spiked drug product as described in *Accuracy* in 〈1225〉), as specified in the sections below. Validation of the dissolution step requires the use of a well-characterized dosage form (e.g., having tight content uniformity and uniform performance). Depending on the parameter of interest, validation of the sample handling and analytical procedure can be performed in situ, e.g., within the dissolution vessel. The validation parameters addressed and the extent of the validation may vary, depending on the phase of development or the intended use for the data.

The acceptance criteria are presented as guidelines only, and may differ for some products. Manufacturers should document the appropriate acceptance criteria for their products in pertinent Standard Operating Procedures (SOPs) or in validation protocols. Other considerations may be important for special dosage forms. Validation studies should be performed across the range of profile time points. For products containing more than a single active ingredient, the dissolution procedure needs to be validated for each active ingredient. It is expected that investigations into filter suitability and the potential for glass adsorption will have been undertaken already (see *1.1 Performing Filter Compatibility*). Validation of these assessments may occur during spiked recovery experiments.

5.1 Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by placebo constituents, other active drugs, or degradants. The placebo consists of all the excipients and coatings, with inks and capsule shells included if appropriate, without the active ingredient. Placebo interference can be evaluated by using a spiked placebo that is prepared by weighing samples of the placebo blend, dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing, and adding a known amount of the drug in solution. It may be preferable to perform this experiment at 37°, comparing the solution to a standard solution at the concentration expected to be encountered during testing, by using the formula:

$$\text{Result} = (A_P/A_S) \times C_S \times (V/L) \times 100$$

A_P = absorbance of the placebo
A_S = absorbance of the standard
C_S = concentration of the standard (mg/mL)
V = volume of the medium (mL)
L = label claim (mg)

The interference should not exceed 2%. Note that for extended-release products, a placebo version of the finished dosage form may be more appropriate than blends because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile, with worst-case interference expected at the later sampling points.

The blank is the dissolution medium without dissolved sample, and it is treated in the same manner as the sample. The effect of the absorbance of the blank at the analytical wavelength should be evaluated. In most cases, the absorbance of the dissolution medium blank may not exceed 1% of the standard solution at the concentration used for analysis. Values >1% should be evaluated on a case-by-case basis.

If the placebo interference exceeds 2%, modification of the method may be necessary. Possible modifications include choosing another wavelength, subtracting baseline using a longer wavelength, transforming absorbance values (e.g., first derivative), and using an alternative analytical technique such as HPLC. Other means for minimizing the placebo interference would be acceptable with appropriate justification. When other active drug substances or significant levels of degradants are present, it is necessary to show that these do not significantly affect the results. One procedure for doing this is to measure the matrix in the presence and absence of the other active drug substance or degradant: any interference should not exceed 2%. Similar approaches may be used if other techniques are used for the analytical finish.

5.2 Linearity and Range

Linearity is typically established by preparing solutions of the drug substance, ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. The solutions may be prepared either using either a standard solution or spiked solution or by the method of standard addition. A minimum of five concentrations is normally used (see ⟨1225⟩). Typically, solutions are made from a common stock if possible. The concentration range may not exceed the linearity limits of the method, including the instrumentation. Organic solvents may be used to enhance drug solubility for the preparation of the linearity standard solutions. However, no more than 5% (v/v) of organic solvent should be present in the final solution unless validated. Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient ($r^2 \geq 0.98$) demonstrates linearity. In addition, the y-intercept must not be importantly different from zero.

The range of the procedure is the interval between the upper and lower concentrations of the drug substance (including these levels) that has been demonstrated to have a suitable level of precision, accuracy, and linearity using the procedure as written.

5.3 Accuracy/Recovery

Accuracy/recovery is typically established by preparing multiple samples containing the drug substance and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. Accuracy/recovery may be done in

conjunction with linearity determination. The method of standard addition can also be used. Before this activity, it is expected that filter assessment will already have been performed, and adsorption of drug onto the glass has also been investigated and ruled out.

Individual solutions may be directly prepared in the dissolution medium. Alternatively, to enhance drug solubility it may be appropriate to prepare a stock solution by dissolving the drug substance in a small amount of organic solvent (typically not exceeding 5% organic solvent in the final dissolution media) and diluting to the final concentration with dissolution medium. An amount of stock solution equivalent to the targeted label claim may be used instead of the drug substance powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts.

The measured recovery is typically 95%–105% of the amount added. Bracketing or matrixing of multiple strengths may be useful. A special case for validation is the *Acid Stage* procedure described in ⟨711⟩, *Delayed-Release Dosage Forms*. The limit of NMT 10% needs to be validated. Recovery experiments for drugs that have low solubility in acidic media may be challenging or impossible to perform and may need to be addressed on a case-by-case basis. If the compound degrades in acid, the validation experiment must address this fact.

5.4 Precision

5.4.1 REPEATABILITY OF ANALYSIS

For the analytical finish, repeatability is evaluated by obtaining replicate measurements of standard and/or spiked placebo/standard addition solutions. It can be determined by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or by using the accuracy or linearity data. ICH guidance, *Validation of Analytical Procedures: Methodology*, recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration. A typical acceptance criterion is an RSD of <2%. The demonstration of the repeatability for the dissolution step is conducted by performing the dissolution step on separate units of a well-characterized dosage form or equivalent composite.

5.4.2 INTERMEDIATE PRECISION/RUGGEDNESS

Assuming that the major contributor to the variance is from the dissolution step, intermediate precision may be evaluated to determine the effects of random events on the precision of the dissolution procedure. This evaluation is typically done later in the development of the drug product and is required for full method validation. For many analytical procedures intermediate precision is typically assessed by determination of contributions to variance and, possibly, by a comparison of means. The use of an experimental matrix design is encouraged for evaluation of intermediate precision because interaction effects may be observed more clearly relative to a single variable experiment. In dissolution testing, a ruggedness approach that compares means alone is often taken to investigate the factors that contribute to intermediate precision. The ruggedness can be evaluated across the range of product strengths. Typical variations to be studied include different days, analysts, and equipment. If possible, ruggedness can be evaluated using a drug product lot if well characterized, for example, by having tight content uniformity and uniform performance, but if this type of lot is not available, a premeasured placebo with active ingredients may be used to investigate the intermediate precision. The use of such a spiked placebo would additionally support the assessment of the contribution of the analytical finish to the observed variability of results.

The dissolution procedure on the same lot of well-characterized dosage form may be run by at least two different analysts from the same laboratory, with each analyst preparing the standard solutions and the medium and following the defined extraction/quantification procedure. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers, and they perform the test on different days. Full profiles are assessed where relevant to the product. This procedure may not be necessary at each strength; instead, bracketing with high and low strengths may be acceptable.

Acceptance criteria for intermediate precision or for ruggedness are predetermined. A typical acceptance criterion for ruggedness is that the difference in the mean value for dissolution results between any two conditions, using the same strength, does not exceed an absolute 10% at time points with <85% dissolved and does not exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

5.4.3 REPRODUCIBILITY

Reproducibility follows the general concepts of intermediate precision, but is performed by two different analysts at different labs.

5.5 Robustness

Evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in development of the drug product and is a requirement for full method validation. It is performed using a well-characterized lot of drug product, for example having tight content uniformity and uniform performance. The number of replicates (typically 3 or 6) is dependent on the intermediate precision. All profile points should be evaluated.

Selection of parameters to be varied depends on the dissolution procedure and analysis type. The parameters may include medium composition (e.g., buffer or surfactant concentration, pH, deaeration), volume, agitation rate, sampling time, and temperature. Statistical analysis of the data generated will help determine the extent to which the parameters must be controlled in the method. The robustness assessment is well suited to Design of Experiments (DoE) methodologies to efficiently investigate the impact of the individual parameters and/or their interaction.

Robustness of analytical finish is referenced in 〈1225〉. HPLC analysis parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

5.6 Stability of Standard and Sample Solutions

The standard solution is stored under conditions that ensure stability. The stability of the standard solution is analyzed over a specified period of time (for at least the time of the entire dissolution procedure), using a freshly prepared standard solution at each time interval for comparison. The acceptable range for standard solution stability is influenced by the concentration and is typically between 98% and 102% at the expected final concentration.

The sample solution is typically stored at room temperature. The sample is analyzed over a specified period of time, using the original sample solution response for comparison. The typical acceptable range for sample solution stability may be between 98% and 102%, compared with the initial analysis of the sample solutions. If the solution is not stable, aspects to consider include temperature (refrigeration may be needed), light protection, and container material (plastic or glass).

The procedure may state that the standards and samples need to be analyzed within a time period demonstrating acceptable standard and sample solution stability.

5.7 Considerations for Automation

Automated methods offer opportunities for increased precision and reproducibility; however, bias may be introduced. In particular, the sampling probe and the sample lines warrant attention as places where inaccuracies may occur. Deviations from the procedure described in 〈711〉, such as resident sampling probes, sampling through the stirring element shaft (hollow-shaft sampling), or fiber-optic probes, should be validated. Other aspects of automation validation may include carryover of residual drug, effect of an in-residence probe, adsorption of drug, and cleaning and/or rinse cycles. Validation is performed using the automated dissolution system including materials. Therefore, any change in materials will require demonstration of suitability based on the validation attributes that are impacted by the change.

Manual and automated procedures should be compared to evaluate the interchangeability of the procedures. This is done by performing two automated runs at each dosage concentration, using all sampling points, compared to manually sampled runs of the same samples. The effect of the in-resident probe cannot be determined by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision if the procedures are to be considered interchangeable. The difference in the mean value for dissolution results between any two conditions using the same strength should not exceed an absolute 10% at time points with <85% dissolved nor exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

Revalidation may be necessary when the automated system is used with different formulations because of the interaction with excipients. Dissolution media containing surfactants or lipids may require additional validation efforts.

6. ACCEPTANCE CRITERIA

The acceptance criteria should be consistent with historical release or stability data. There is an expectation that acceptable batches will have results that fall within the acceptance criteria and that all manufactured batches should have similar dissolution behavior, thus highlighting the importance of having a method that is not highly variable. The acceptance criteria and time point(s), therefore, should discriminate between an acceptable and an unacceptable batch. In addition, the dissolution test results are viewed as a link to the pivotal clinical trial batches. When changes in dissolution rate have been shown to affect bioavailability significantly, the dissolution test and acceptance criterion should distinguish batches with unacceptable bioavailability *(19)*. Likewise, when changes in the formulation and manufacturing process significantly affect dissolution and such changes are not controlled by another aspect of the specification, the dissolution test and criteria should distinguish these changes.

General Chapters

6.1 Immediate-Release Dosage Forms

Although release and stability data are collected during dosage form development, it is common to record the entire dissolution profile or the amount of drug dissolved at specified intervals, such as 10, 20, 30, 40, 50, and 60 min or 15, 30, 45, and 60 min. At registration, dissolution for an immediate-release tablet usually becomes a single-point test. The acceptance criterion and test time are established by evaluating the dissolution profile data. The acceptance criterion for a dissolution test is a function of *Q*, which is expressed as a percentage of label claim of drug dissolved at a specified time. Typical *Q* values are in the range of 75%–80% dissolved. *Q* values in excess of 80% are not generally used because allowance needs to be made for assay and content uniformity ranges.

6.2 Delayed-Release Dosage Forms

The discussion about dissolution of delayed-release dosage forms in ⟨711⟩ focuses on enteric-coated dosage forms, which is the most common delayed-release dosage form. A dissolution test for a delayed-release tablet or capsule is a two-part test, and each part has acceptance criteria. First, the dosage forms are exposed to an acid medium, followed by exposure to a buffer medium. To ensure that the enteric coating performs properly, a "NMT" acceptance criterion is indicated in ⟨711⟩ for the acid stage. The medium used for an acid stage is usually 0.1 N HCl, and the duration of this stage is typically 2 h. The dosage forms are then exposed to a buffer medium, usually 0.05 M phosphate buffer at pH 6.8, but other buffers and pH targets may be used if justified. The duration of the buffer stage is usually 45 min for compendial tests, but this duration may vary, depending on the drug product. As with immediate-release dosage forms, a *Q* value and time point are determined by evaluating the entire dissolution profile.

6.3 Extended-Release Dosage Forms

A dissolution test for an extended-release dosage form is generally similar to that used for an immediate- or delayed-release drug product, except that the duration of the test is longer, and at least three time points are specified for pharmacopeial purposes *(20)*. Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug *(20)*. The time points for the test should be determined by evaluating the dissolution profile across the desired test duration. Often, additional time points are obtained during dosage form development to aid with selecting the appropriate time points for the specification or monograph.

As with an immediate- or delayed-release drug product, the acceptance criteria and time points for an extended-release drug product should discriminate between an acceptable and an unacceptable batch. The acceptance criteria for the first stage of testing (L_1) should be established on the basis of available batch data *(19,20)*. If human bioavailability data are available for formulations exhibiting different release rates, then an in vitro/in vivo relationship may be used to establish acceptance criteria *(19,20)*. Acceptance criteria for the second (L_2) and third (L_3) stages are derived from the L_1 criteria using *Acceptance Table 2* in ⟨711⟩.

6.4 Multiple Dissolution Tests

Typically, monographs for extended-release dosage forms contain multiple dissolution tests representing specific products. In accordance with *General Notices and Requirements 4.10.10*, the appropriate test, if not *Test 1*, is indicated on the product labeling. For example, the *USP* monograph for *Oxycodone Hydrochloride Extended-Release Tablets (21)* lists two dissolution tests, each of which has either three or four time points. If the Tablets are analyzed using *Test 2* and the dissolution results comply with the criteria provided in the monograph, the labeling for Tablets can indicate that the Tablets meet USP *Dissolution Test 2*. Multiple dissolution tests also can be found in monographs for immediate- and delayed-release dosage forms. For example, the *USP* monographs for *Levothyroxine Sodium Tablets* and *Pantoprazole Sodium Delayed-Release Tablets* provide four dissolution tests *(22,23)*.

6.5 Interpretation of Dissolution Results

The *Interpretation* section of ⟨711⟩ discusses immediate-, delayed-, and extended-release dosage forms. The discussion for each of these release patterns is expanded here with examples to assist with applying the criteria during the various stages of testing. Understanding how these criteria are applied will assist in setting appropriate acceptance criteria.

6.5.1 IMMEDIATE-RELEASE DOSAGE FORMS

Once the *Q* value is established, the dissolution test is a staged test of three levels. In the first level of testing called S_1, six dosage forms are tested. Each dosage form must be *Q* + 5% (absolute percentage points) dissolved at a specified time. For example, the time and tolerances in a monograph would be:

Time: 30 min

Tolerances: NLT 80% (*Q*) of the labeled amount of "drug substance" is dissolved.

If the *Q* value for a 200-mg label claim (LC) immediate-release tablet is specified as 80% and the time point is 30 min, then NLT 85% LC (170 mg) of the drug substance in each tablet must be dissolved at 30 min.

If this criterion is not met, then 6 additional tablets are tested at level 2 (S_2). To pass the S_2 acceptance criteria, the average of all 12 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example), and no tablet has less than *Q* – 15% (65% LC; 130 mg in the above example).

If these criteria are not met, then level 3 or S_3 testing must be performed by testing 12 additional tablets. To pass S_3, the average of all 24 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example). Two additional criteria must be met as well: 1) no more than 2 tablets are less than *Q* – 15% (65% LC; 130 mg in the above example), and 2) no tablet is less than *Q* – 25% dissolved (55% LC; 110 mg in the above example.)

6.5.2 DELAYED-RELEASE DOSAGE FORMS

An aliquot of the acid medium from each vessel is analyzed at the end of the acid stage. For the acid stage, the acceptance criteria have three levels. Level 1 (A_1) testing is passed if no individual value exceeds 10% dissolved. If the A_1 criteria are not met, then the dissolution test is performed on 6 additional dosage forms for level 2 (A_2) testing. Level A_2 criteria are passed if the average of all 12 dosage forms in the acid stage is NMT 10% dissolved and if no individual dosage form is more than 25% dissolved. Level 3 testing is performed if the A_2 criteria are not met. The A_3 criteria are passed if the average of all 24 dosage forms in the acid stage is NMT 10% dissolved and if no individual tablet is more than 25% dissolved. For the special case in which the solubility of the drug in an acidic medium because of conversion to the free acid is too low to support an acceptance criterion of not more than 10% the drug product should be exposed to the acid stage for the defined duration and then exposed to the buffered medium. Alternate acceptance criteria for the acid stage based on drug solubility may be justified.

For delayed-release dosage forms, the total percentage dissolved is determined by adding the measured amounts in the acid and buffer phases for each individual dosage form. These calculated values are then compared to staged acceptance criteria (B_1, B_2, and B_3) that are based on a *Q* value. The B_1, B_2, and B_3 criteria are identical to those for the immediate release S_1, S_2, and S_3 criteria.

6.5.3 EXTENDED-RELEASE DOSAGE FORMS

In the following hypothetical example, which is used to describe the criteria for an extended-release dosage form, the time points are 1, 4, and 8 h. The acceptance range for each time point is as follows:

- Between 24% and 44% LC drug substance dissolved at 1 h
- Between 56% and 76% LC drug substance dissolved at 4 h
- NLT 85% LC drug substance dissolved at 8 h.

Acceptance ranges are often expressed in tabular form in the *USP–NF* (see *Table 3*):

Table 3. L_1 Criteria

Time (h)	Amount Dissolved
1	24%–44%
4	56%–76%
8	NLT 85%

Six tablets are analyzed at Level 1 (L_1); acceptance criteria are met if no individual value lies outside each of the stated ranges, and no individual value is less than the percentage specified for the final time point. If the L_1 criteria are not met, then 6 additional tablets are analyzed at level 2 (L_2). The L_2 criteria are met if these three conditions are met:

1. The average value of the 12 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. None of the 12 tablets is >10% of the labeled content outside each of the stated ranges.
3. None of the 12 tablets is >10% of the labeled content below the stated amount at the final test time.

For the above example, the L_2 acceptance criteria for the 12 tablets (see *Table 4*) are as follows:

Table 4. L_2 Criteria

	1 h	4 h	8 h
Average	24%–44%	56%–76%	NLT 85%

Table 4. L_2 Criteria *(Continued)*

	1 h	4 h	8 h
Individual tablets	14%–54%	46%–86%	NLT 75%

If the L_2 criteria are not met, then 12 additional tablets are tested at level 3 (L_3). The L_3 criteria are met if these five conditions are met:

1. The average value of the 24 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. NMT 2 of the 24 tablets are >10% of labeled content outside each of the stated ranges.
3. NMT 2 of the 24 tablets are >10% of the labeled content below the stated amount at the final test time.
4. None of the 24 tablets is >20% of the labeled content outside each of the stated ranges.
5. None of the 24 tablets is >20% of the labeled content below the stated amount at the final test time.

The L_3 acceptance criteria for the 24 tablets in the above example are summarized in *Table 5*:

Table 5. L_3 Criteria

	1 h	4 h	8 h
Average	24%–44%	56%–76%	NLT 85%
Individual Tablets	NMT 2 tablets are outside the range of 14%–54%, and no individual tablet is outside the range of 4%–64%	NMT 2 tablets are outside the range of 46%–86%, and no individual tablet is outside the range of 36%–96%	NMT 2 tablets release <75% and no individual tablet releases <65%

REFERENCES

1. FDA. Recommended dissolution methods. http://www.accessdata.fda.gov/scripts/cder/dissolution/index.cfm. Accessed 10 Sep 2014.
2. Mihali C, Oprea G, Cical E. Determination of critical micelar concentration of anionic surfactants using surfactants—sensible electrodes. *Chem Bull "Politehnica" Univ (Timişoara).* 2008; 53(67):1–2.
3. Stippler ES. Biorelevant dissolution test methods to assess bioequivalence of drug products [PhD dissertation]. Frankfurt: Johann Wolfgang Goethe University; 2004.
4. Koga K, Kusawake Y, Ito Y, Sugioka N, Shibata N, Takeda K. Enhancing mechanism of Labrasol on intestinal membrane permeability of the hydrophilic drug gentamicin sulfate. *Eur J Pharm Biopharm.* 2006; 64(1):82–91.
5. Neugebauer JM. Detergents: an overview. *Methods Enzymol.* 1990; 182:239–253.
6. Harris ELV, Angal S, editors. *Protein Purification Applications: A Practical Approach.* Oxford, England: IRL Press at Oxford University Press; 1990; 71.
7. Helenius A, McCaslin DR, Fries E, Tanford C. Properties of detergents. *Methods Enzymol.* 1979; 56:734–749.
8. Kassel D. Properties of cremophore EL micelles probed by fluorescence. *Photochem Photobiol.* 1992; 56(4):447–451.
9. Rowe RC, Sheskey PJ, Quinn ME, editors. *Handbook of Pharmaceutical Excipients.* 6th ed. London: APhA; 2003.
10. Rosenthal KS, Koussale F. Critical micelle concentration determination of non-ionic detergents with coomassie brilliant blue G-250. *Anal Chem.* 1983; 55:1115–1117.
11. Herrman KW. Non-ionic–cationic micellar properties of dimethyldodecylamine oxide. *J Phys Chem.* 1962; 66:295–300.
12. Leeson LJ, Adair D, Clevenger H, Chiang N. The in vitro development of extended-release solid oral dosage forms. *J Pharmacokinet Biopharm.* 1985; 13(5):493–514.
13. Dressman JB, Amidon GL, Reppas C, Shah VP. Dissolution testing as a prognostic tool for oral drug absorption: immediate release dosage forms. *Pharm Res.* 1998; 15(1):11–22.
14. FDA. Guidance for industry. Waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. August 2000. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070246.pdf. Accessed 14 Jun 2013.
15. Kakhi M. Classification of the flow regimes in the flow-through cell. *Eur J Pharm Sci.* 2009; 37(5):531–544.
16. FDA. Guidance for industry. Dissolution testing of immediate release solid oral dosage forms. 1997. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/UCM070237.pdf. Accessed 10 Sep 2014.
17. FDA. 21 CFR Part 11. April 2012. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=11. Accessed 14 Jun 2013.
18. ICH. Q2(R1). Validation of analytical procedures. 1996. http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html. Accessed 17 Jun 2013.
19. ICH. Q6A. Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances. October 1999. http://www.ich.org/products/guidelines/quality/quality-single/article/specifications-test-procedures-and-acceptance-criteria-for-new-drug-substances-and-new-drug-produc.html. Accessed 14 Jun 2013.
20. FDA. Guidance for industry. Extended release oral dosage forms: development, evaluation, and application of in vitro/in vivo correlations. September 1997. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070239.pdf. Accessed 14 Jun 2013.
21. USP. *USP 36–NF 31, Oxycodone Hydrochloride Extended-Release Tablets.* Rockville, MD: USP; 2013:4642–4644.

22. USP. *USP 36–NF 31, Levothyroxine Sodium Tablets*. Rockville, MD: USP; 2013:4109–4110.
23. USP. *USP 36–NF 31, Pantoprazole Sodium Delayed-Release Tablets*. Rockville, MD: USP; 2013:4682–4686.

⟨1094⟩ CAPSULES—DISSOLUTION TESTING AND RELATED QUALITY ATTRIBUTES

1. INTRODUCTION

This general information chapter provides approaches for the development of dissolution test procedures for capsules, which are not provided by *Dissolution* ⟨711⟩, *Drug Release* ⟨724⟩, *The Dissolution Procedure: Development and Validation* ⟨1092⟩, and *Disintegration and Dissolution of Dietary Supplements* ⟨2040⟩. The chapter also discusses quality attributes associated with capsules that may affect the outcome of the dissolution testing.

1.1 Types of Capsules

Capsules can be classified as two main types based on the physical characteristics of the shell: soft capsules and hard capsules. For the purpose of this chapter, soft-shell capsules and hard-shell capsules are referred to as softgels and hardgels, respectively. Softgels have a thicker shell and typically exhibit a higher degree of elasticity because of the added plasticizer and have slightly longer rupture time when compared with hardgels. By comparison, hardgel capsules have a thinner and more rigid shell than do softgel capsules. Both softgels and hardgels are composed of a polymer, e.g., gelatin, starch, or a cellulose derivative such as hypromellose (HPMC) or other polymers, a plasticizer, and water. For hardgels, water acts as the plasticizer, whereas softgels contain high-boiling-point polyols such as glycerol or sorbitol as plasticizer, and also contain water. Although many parameters affect the physical and chemical properties of the shell, the ratio of polymer to plasticizer primarily determines the rigidity, brittleness, and dissolution performance of the shell.

Capsules can also be characterized by the chemical properties of the fill material (hydrophobic-based versus hydrophilic-based fill materials) or by the physical properties of the fill material (solution versus dispersion versus solid). Hydrophobic solutions include neat oils, combinations of miscible oils, or active ingredients dissolved in oil vehicles. Hydrophobic dispersions include active ingredients dispersed or suspended in oil or in oil-wax mixtures. The latter often are termed semisolids. Hydrophilic solutions can be neat liquids, combinations of water-miscible liquids, or active ingredients dissolved in water-miscible vehicles. Hydrophilic dispersions or suspensions include active ingredients dispersed or suspended in hydrophilic vehicles such as polyethylene glycol. Solid fill materials consist of mixtures of excipients and active ingredients whose properties like hydrophilicity/hydrophobicity, polymorphism, particle size, etc., drive the dissolution behavior.

1.2 Manufacturing and Packaging Issues That Can Affect Dissolution Testing

A number of issues affect the development of a dissolution procedure and the dissolution behavior for capsules, including:
- Properties of capsule shell material
- Properties of the fill material
- Interaction between capsule shell material and fill material

Gelatin is a hygroscopic material, and its moisture content affects the properties of hard and soft gelatin capsules. Since certain excipients are known hygroscopic agents, it is particularly important to monitor the mechanical properties of gelatin capsules stored under various conditions of temperature and relative humidity. The factors that can affect the capsule properties include: moisture exchange between the shell and the fill material, which potentially can create brittleness in the gelatin shell; and chemical interactions between the fill material and gelatin, which can result in gelatin cross-linking.

The potential for aldehydic impurities and formation of degradants and degradation of the active ingredients or excipients in the formulation should be investigated during product development by means of stability studies (aldehydes contribute to cross-linking; see section *2. Cross-Linking in Gelatin Capsules*). Understanding the possible routes of aldehyde or ketone formation, and possible sources of aldehyde, helps to predict capsule behavior. The rate of cooling and drying may modify the characteristics of the active ingredient release from the matrix. The possibility of migration of the active ingredient into the capsule shell, particularly when the softgel is made of gelatin, should be investigated for its impact on dissolution testing.

Capsule shells can become reactive depending on the storage conditions. Product packaging and storage conditions are chosen to prevent adverse effects on capsule quality. The ingress of moisture and/or oxygen into the packaging and through the capsule shell, as well as the rate of ingress, can affect the final shelf life for the product. In some cases, an increase in water content of the capsule shell may produce a measurable reduction in the dissolution time because a moistened product may facilitate polymer hydration.

Due to the water content of gelatin shell (usually between 13% and 16%), this type of capsule behaves as a moisture reservoir which may affect the stability of humidity-sensitive active ingredients.

When a capsule is immersed in an aqueous medium, the water permeates the walls, the polymer becomes hydrated, and swells. When fully hydrated, the shell starts to dissolve. The amount of time it takes for water to penetrate capsule shells varies, depending on the nature of the capsules shell and other factors. This time has been reported to be approximately 40 s for gelatin capsules and about 3 min for HPMC capsules. This delay may be significant only for dissolution testing of immediate-release dosage forms, while it should have minor or no impact on dissolution testing of modified-release formulations.

2. CROSS-LINKING IN GELATIN CAPSULES

Cross-linking involves the formation of chemical links stronger than the simple hydrogen and ionic bonding between gelatin chains and affects the thermal reversibility of the sol-gel transition of gelatin in the shell. Cross-linking can be caused by agents present in the capsule fill that react with gelatin molecules, resulting in the formation of a pellicle on the internal surface of the shell. Less often, a pellicle may form on the external surface of the shell arising from reactive agents present in, or derived from the intermediate of final packaging components.

A pellicle is a thin, water insoluble clear membrane of cross-linked protein on the inner or outer surface of the capsule that prevents the capsule fill from being released. Cross-linking is evidenced by the observation of a thin membrane or a gelatinous mass during dissolution testing because the pellicle itself may be difficult to observe.

Cross-linking can also be caused by agents or impurities present in the shell, thereby rendering the entire shell matrix insoluble under conditions that normally would dissolve the gelatin shell. One of the strongest and most common types of cross-linking involves the covalent bonding of the amine group of a lysine side chain of one gelatin molecule to a similar amine group on another molecule. This reaction is typically caused by trace amounts of reactive aldehydes. Formaldehyde, glutaraldehyde, glyoxal, and reducing sugars are the most common cross-linking agents. The covalent bonding produced with this type of cross-linking is, for all practical purposes, irreversible, and dissolution of the shell must involve the breaking of other bonds such as the enzyme-mediated breaking of the peptide bonds in the protein chains.

Gelatin that is chemically modified, e.g., by the addition of succinic acid groups to the lysine side chains, can prevent or at least hinder aldehyde-mediated cross-linking. A weaker type of cross-linking involves complexation of free carboxylic acid groups from two different gelatin molecules with trivalent metal ions such as Fe^{3+} and Al^{3+}. These cations can be found in some of the dyes used as colorants or as low-level contaminants of excipients. For higher-Bloom gelatin (see section *6.1.1 Gelatin*), which typically is considered higher quality, cross-linking occurs more readily because fewer links are needed to join greater lengths of gelatin chains.

It is extremely important to know and understand the product formulation to identify possible sources of cross-linking agents and take measures to eliminate or minimize their role in promoting cross-linking. This knowledge can help in the case of post-approval changes in the formulation and/or packaging material.

Common causes of cross-linking include the following:

- Aldehydes that are present in the active ingredient, excipients, or packaging materials (e.g., in residual solvents); or that may be formed in-situ during storage
- High humidity (leading to higher oxygen permeability)
- Substances that facilitate a cross-linking reaction
- Substances that promote decomposition of stabilizer in corn starch (hexamethylenetetramine), resulting in the formation of ammonia and formaldehyde, which cause the cross-linking reaction
- Rayon coilers that contain an aldehyde functional group (furfural)
- Polyethylene glycol may contain peroxides and aldehydes
- UV light, especially with high heat and humidity
- Aldehyde formation promoted by elevated temperatures

Dissolution testing of cross-linked capsules can result in slower release of the drug or no release at all. On rare occasions, if there are defects in the liquid-filled capsule seam, the capsule can rupture at the seam even in the presence of cross-linking in the gelatin, resulting in an early release of the capsule fill in the dissolution medium. The degree of cross-linking is not uniform within one capsule or among different capsules. As consequence, there is a higher variability in the dissolution results if the gelatin capsules are cross-linked. Enzymes can be added to the dissolution medium to overcome this problem (see *Dissolution* ⟨711⟩). Enzymes should not be used in the absence of such evidence.

3. DISSOLUTION PROCEDURE DEVELOPMENT

Design of a procedure for dissolution testing of capsules depends on the product formulation. The composition of the fill, the solubility of the active ingredient(s) in the fill, and the dispersion of the fill into the dissolution media all have an effect on the dissolution behavior of a given fill formula. For example, a formulation that contains a lipid-soluble active ingredient with hydrophobic excipients and a melting point in excess of 37° will likely not release the active ingredient into solution in aqueous media in a timeframe that is consistent with the expectations for immediate-release dosage forms. By contrast, a freely water-miscible active ingredient dissolved or dispersed in a water-soluble or water-dispersible fill formula at room temperature will be released into solution very soon after the shell ruptures and the dosage form releases the fill material into the media.

The specific design of the formulation and the target release profile should be known when developing a dissolution method for any given product. For lipid-based formulations that often are less dense than aqueous media, the release of the fill from

the capsule may be driven by buoyancy. If the formulation has been designed to be self-emulsifying or self-microemulsifying, the formulation will efficiently disperse into most aqueous media. Formulations such as those that consist simply of a triglyceride with no additional co-solvent or emulsifier will rapidly float to the dissolution medium surface in the vessel. In this situation the quantitative results from a dissolution test provide the rate of drug partition from this floating layer. The apparatus selection is perhaps the most critical step for these types of capsules because the efficiency with which the capsule contents mix with dissolution media is highly influenced by the hydrodynamics of the agitation. Once the apparatus has been selected, other variables to consider in developing a dissolution test are rotation speed, dip rate or flow rate, surfactant/solubility enhancer type and concentration, and medium volume and pH (see ⟨711⟩, ⟨1092⟩, and ⟨2040⟩).

To better guide the dosage form design it may be useful to consider other characteristics of the product such as solubility of the active ingredient(s) in different media, intrinsic dissolution of the active ingredient(s) (see *Apparent Intrinsic Dissolution—Dissolution Testing Procedures for Rotating Disk and Stationary Disk* ⟨1087⟩), dispersibility, globule size for emulsions, micelle formation, digestion, precipitation on dilution in media, phase behavior studies, and burst tests to detect gelatin cross-linking.

The rupture test may be a useful tool in the early steps of the development and evaluation of the formulation, especially if the active ingredient belongs to Biopharmaceutics Classification System Class 1 (see *FDA Guidance for Industry—Waiver of In vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*) and is formulated in a water-miscible fill. For this purpose, rupture is considered to occur when the capsule shell is breached, exposing the fill contents. If the rupture of the capsule shell can be correlated with dissolution data, then rupture can be explored as a possible alternative to the dissolution test.

3.1 Apparatus

As is the case with other oral solid dosage forms, USP *Apparatus 1* (basket), USP *Apparatus 2* (paddles), USP *Apparatus 3* (reciprocating cylinder), and USP *Apparatus 4* (flow-through cell) are most often chosen as the dissolution apparatus for capsules (see ⟨711⟩ and ⟨2040⟩). However, several specific benefits and drawbacks must be considered in selecting and using the dissolution apparatus for capsules:

- Capsules may be filled with a material that has a specific gravity less than that of water. Therefore, the capsules may float in an aqueous dissolution medium;
- Instead of dissolving completely during the dissolution test, the capsule shell may soften and disintegrate into a sticky or waxy mass that can adhere to any point in the dissolution vessel and in different areas in different vessels, generating high variability in the results.
- The capsule fill material may form a film on the surface of the dissolution medium during the course of the test.

The capsule dissolution process involves three stages: (1) rupture of the capsule shell, (2) release and dispersion of the capsule fill material, and (3) dissolution of the active ingredient(s) in the medium. Each dissolution apparatus referred to previously can achieve these three stages, but they may cause different hydrodynamic effect upon capsules as on any other dosage form as well.

3.1.1 USP APPARATUS 1 (BASKET)

This apparatus has the advantage of enclosing the capsules, preventing them from floating freely in the medium. For certain capsules, however, baskets may not be suitable. As the capsule ruptures, the material from the capsule shell may clog the basket's mesh, and for hydrophobic fill materials the oil phase released from the capsule may not disperse into fine enough droplets in the basket to efficiently pass through the mesh. A larger-size mesh may be needed to overcome these limitations.

3.1.2 USP APPARATUS 2 (PADDLES)

This apparatus does not have a mesh that can clog, but it does not prevent the capsules from floating. In these instances, wire coils can be wound around the capsules, or commercially available sinkers can be used to encase the capsules and hold them on the bottom of the vessel, allowing the fill to become exposed to more of the medium (see *Apparatus/Agitation, Sinkers* in ⟨1092⟩). Sinkers can also be used to prevent the capsule from sticking to the vessel walls and to provide better contact with the dissolution medium even if the capsule does not float and remains at the bottom of the vessel. The shape and size of the sinker can play an important role in the dissolution profile and should be selected carefully. The swelling, that occurs when the capsule is placed in contact with the dissolution medium, should be considered when defining the size of the sinker to be used.

3.1.3 USP APPARATUS 3 (RECIPROCATING CYLINDER)

This apparatus, like *Apparatus 1*, encloses the capsules. The mesh at the bottom of the cylinder, however, may become clogged with undissolved shell. Changing the mesh size may alleviate this problem. The different mechanism of agitation of *Apparatus 3* provides very different hydrodynamics compared with *Apparatus 1* and *2*. This characteristic may assist in dispersing hydrophobic droplets, avoiding the formation of layers and some problems caused by the buoyancy of the capsule. In the case

of *Apparatus 3*, a change to different media during the course of the dissolution experiment is possible. This allows dissolution profiles to be determined at different pH values, which is useful for targeted-action or modified-release dosage forms. For cases where the active ingredient has been dispersed but not dissolved, this apparatus may not be a good choice since significant sample loss will occur when moving the cylinder from one tube to the other. However, USP *Apparatus 3* has a tendency to generate foam when surfactants are added to the media. In this situation, an appropriate antifoaming agent may be added to the medium.

An alternative to *Apparatus 3* is the disintegration apparatus described in *Disintegration* ⟨701⟩. The use of disks should be avoided because of clogging. If the discs are not used, the capsule will float and may not be uniformly wetted. This apparatus offers more turbulent conditions that could be useful in the dispersion of hydrophobic filling, avoiding the formation of layers and counteracting the buoyancy of the capsule.

3.1.4 USP APPARATUS 4 (FLOW-THROUGH CELL)

This apparatus also encloses the capsules and has a filter. Several types of cells are available depending upon the application. The use of the flow-through cell designed for lipid-filled softgels may be useful for certain types of formulations (see *Figure 2* in ⟨2040⟩). Use of *Apparatus 4* also makes it possible to change to different media and to alter the flow during the course of the dissolution experiment. In addition, this equipment can be set up for low or high volumes of media that can be used with low-strength products and with poorly soluble active ingredient(s), respectively.

Other apparatus may be considered with proper justification, with each candidate apparatus evaluated for variations in media composition, media volume, agitation or flow rate, and other test parameters to determine what effect each has on the dissolution performance of the product.

General Chapters

3.2 Medium

General recommendations for the selection of appropriate dissolution media can be found in chapters ⟨1092⟩ and ⟨2040⟩, *FDA Guidance for Industry—Dissolution Testing of Immediate-Release Solid Oral Dosage Forms* and *FDA Guidance for Industry—Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In vitro/In vivo Correlations*.

Characteristics of the dissolution medium that may affect the opening or rupture of the capsule shell, the release and dispersion of the capsule fill material, and the dissolution of the active ingredient(s) are discussed in the following sections.

3.2.1 ACHIEVING SINK CONDITIONS

As in the development of any effective dissolution procedure, the medium preferably, but not necessarily, should provide sink conditions for the active ingredient(s) in an environment that ensures suitable stability and, preferably, is physiologically relevant for the product (see ⟨1092⟩).

If the fill is water-soluble or, at least, readily dispersible in an aqueous medium, and if the active ingredient itself is also soluble, sink conditions may be achieved in a manner comparable to that for any other solid oral dosage form. Liquid-filled capsules, however, may contain either a matrix or active ingredient (or both) that are hydrophobic or water-insoluble. In this case, a medium with surfactants may be needed (see below). The use of organic co-solvents to improve sink conditions is discouraged and should be employed only as a last resort and at a minimum amount with appropriate justification. In practice, the presence of organic solvents in the medium may inhibit the dissolution of the shell.

In addition to dispersing/dissolving the matrix and active ingredient(s), the medium must neither interfere with the activity of any enzymes used nor negatively interact with the capsule shell or formulation. Developing a suitable medium for hydrophobic systems therefore may require considerable experimentation. For example, using testing from ⟨711⟩, the addition of enzymes is required when the gelatin capsule fails the test due to pellicle formation. When certain surfactants are used (e.g., sodium lauryl sulfate), the enzyme may be deactivated, depending on the type and concentration of surfactant used. In this case, a pretreatment period with the proteolytic enzyme followed by a later addition of the surfactant to the medium may be considered. The time of this pre-treatment should be as short as possible. In most cases, it does not exceed 15 min. The pre-treatment period is included in the total time of the dissolution testing.

To establish a suitable medium, several different dissolution media should be evaluated to identify the one that achieves appropriate sink conditions with the lowest quantity of solubilizing/dispersing agent. The effect of pH, ionic strength, buffer counter-ion and/or co-solvent on active ingredient solubility and enzyme activity, particularly for hydrophobic active ingredients, in addition to the relative partitioning of the active ingredient between the matrix and medium, must also be evaluated.

3.2.2 USE OF SURFACTANTS/DISPERSING AGENTS/SOLUBILITY ENHANCERS

Surfactants, dispersing agents, or solubility enhancers may be used in the dissolution medium when the capsule fill, the active ingredient, or both, are hydrophobic or water-insoluble. They may also be used if the media described in ⟨1092⟩ and in the FDA guidance are ineffective in dispersing the capsule fill or in achieving proper sink conditions for the active ingredient.

Surfactants, dispersing agents, and solubility enhancers include:

- Anionic:
  - — Sodium dodecyl sulfate (SDS, or sodium lauryl sulfate, or SLS)
  - — Bile salts (sodium deoxycholate, sodium cholate)
- Cationic:
  - — Cetyltrimethylammonium bromide (CTAB)
  - — Hexadecyltrimethylammonium bromide (HTAB)
  - — Methylbenzethonium chloride (Hyamine™)
- Nonionic:
  - — Polysorbates (Tween™)
  - — Polyoxyethylene sorbitan esters
  - — Octoxynol (Triton X100™)
  - — *N,N*-dimethyldodecylamide-*N*-oxide
  - — Brij 721
  - — Polyoxyl castor oil (Cremophor™)
  - — Nonylphenol ethoxylate (Tergitol™)
  - — Cyclodextrins
  - — Polyoxyl 10 lauryl ether
- Zwitterion
  - — Lauryl dimethyl amine oxide (dodecyldimethylamine oxide, DDAO)
  - — Lecithin

Although useful as solubilizing agents, surfactants should be used cautiously because they can interact with the gelatin in the capsule shell and can hinder disintegration or dissolution. They can also inhibit enzymes that may be used to hydrolyze the gelatin shell and/or the fill.

One of the most common anionic surfactants used for dissolution testing of tablets, SLS, exhibits both of these adverse effects, particularly at lower pH (e.g., in simulated gastric fluid). In addition, SLS forms insoluble precipitates in the presence of potassium ions. SLS is one of the surfactants less compatible with enzymes. Only a very high quality grade of SLS should be employed in dissolution media because of the potential interference of its impurities in the quantitation step in dissolution testing. Therefore, the use of SLS should be considered only in the absence of other alternatives.

Other anionic or cationic surfactants have also been shown to affect gelatin solubility, and the extent of these interactions should be considered in the selection of the dissolution medium. Cationic surfactants should be avoided in formulations that contain fatty acids because the combination potentially forms insoluble precipitates.

3.2.3 USE OF ENZYMES

Proteolytic enzymes such as pepsin (at low pH) or pancreatin (at pH ≥6.8) may be used when the gelatin capsule shell does not dissolve in ordinary aqueous media because of cross-linking, as previously discussed. Pancreatin has the advantage of also possessing lipase activity, which makes it useful when the capsule fill material is a triglyceride.

Tests should be carried out to ensure that the enzymes used in the dissolution testing do not adversely interact with the formulation. The dissolution medium should also be optimized (e.g., by adjusting the pH, ionic strength, etc.) so that it preserves enzymatic activity while maintaining sink conditions for the active ingredient. If surfactants are also used, their potential adverse effect on enzyme activity must be evaluated.

3.2.4 PH

In addition to establishing a drug pH-solubility profile during the dissolution development, the following specific issues should also be evaluated:

- The effect of media pH on the swelling or dissolution of the capsule based on the type of gelatin used in the product: either Type A (for which the medium pH typically is 7–9) or Type B (for which the medium pH typically is 4.7–5.4)
- The need for enzymes may influence the selection of a specific pH range in order to be adequate for the active ingredient solubility and stability and to minimize effects on enzyme activity.

3.3 Cross-Linking in Gelatin Capsules

As previously discussed, cross-linking results in the formation of pellicles on the internal or external surface of the shell. Depending on the extent of the cross-linking and the structural integrity of the shell, this pellicle may delay or prevent the release of the fill and the dissolution of active ingredient(s) during the test.

Pellicles often are difficult to dissolve and typically require the use of proteolytic enzymes in the medium to achieve dissolution.

Gelatin also forms ionic cross-links (salt bridges), typically between carboxylate (anionic) and ammonium (cationic) side chains of the amino acids comprising the polypeptide chain. Although they can inhibit gelatin dissolution, ionic cross-links are

much weaker than covalent cross-links and may be disrupted by changing the ionic strength or pH of the medium or by using an enzyme.

3.3.1 FORCED CROSS-LINKING

During product and dissolution method development, forced formation of cross-linking in the gelatin capsule may be useful to establish the type and amount of enzyme that will be used in the test, and to better understand the behavior of the formulation in the dissolution medium. This can be achieved by spiking excipients with known amounts of formaldehyde or other cross-linking agents, or exposing the capsules to high humidity.

3.4 Sampling

Establishing the proper sampling technique and location for capsules follows the procedures described in chapters ⟨711⟩ and ⟨1092⟩. In the case of hydrophobic liquid filled capsules, the fill material typically forms a film on the surface of the dissolution medium during the course of the test, sampling must be performed in such a way that the cannula penetrates the oily layer without being clogged. In addition to standard validation and compatibility studies, care must be taken when a filter is used to ensure that it does not become clogged with oil or undissolved capsule shell material when the sample is taken. Similar considerations apply to automated sampling equipment because filters and transfer lines may become obstructed during sampling. To address this issue, the use of surfactants and/or enzymes in dissolution media may be needed to better solubilize the capsule shell and fill. Another point to be considered in the case of automated sampling equipment is the probe. If it remains inside the medium during the test, it could perturb the oily layer and possibly influence the hydrodynamics and change the dissolution profile. An alternative approach is to remove the probe and to introduce it just at the time of the sampling. The sampling method should be validated for each product.

3.5 Quantitation

Like dissolution samples from other oral solid dosage forms, dissolution samples from capsules can be quantitated using chromatographic, spectrophotometric, tandem chromatography-mass spectrometry, and other techniques after adequate method development and validation (see ⟨1092⟩ and where special considerations are described as follows under section *4. Method Validation*).

4. METHOD VALIDATION

In addition to the general method validation parameters discussed in ⟨1092⟩, the following performance characteristics may be evaluated:

- Effect of pH and ionic strength on drug solubility and on enzyme activity, if enzymes are used
- For hydrophobic active ingredient(s), the relative partitioning of the active ingredient(s) between the matrix and the medium, as well as the potential for an adverse effect on release into the aqueous medium
- For chromatographic procedures, the potential adverse effect of the surfactant, if used, on the chromatographic separation; as part of the robustness study, evaluation of different concentrations of surfactant, different surfactant types, interaction with buffer salts, etc.
- For spectrophotometric procedures, absorbance from the potential contribution of the capsule shell should be evaluated
- Potential adverse effects of the surfactant or enzyme, if present, on the lifetime of the chromatographic column
- Qualification of the sampling procedure to prevent clogging of the cannula, transfer tubing, or filters
- Validation of the second tier dissolution test
    - —pre-soaking time
    - —compatibility of any surfactants present with the enzymes
    - —verification that the conditions of the second tier test do not affect the dissolution profile when compared to the first tier test

5. SUGGESTIONS FOR STARTING POINTS

Based on the considerations discussed above, possible starting points for establishing a dissolution test for capsules based on the solubility characteristics of the fill and the active ingredient(s) are the following:

Possible formulations:

1. Hydrophilic fill/active ingredient is soluble in aqueous media
2. Hydrophobic fill/active ingredient is poorly soluble in aqueous media
3. Hydrophilic fill/active ingredient is poorly soluble in aqueous media
4. Hydrophobic fill/active ingredient is soluble in aqueous media

5.1 Medium

Evaluate the solubility of the active ingredient(s) in aqueous media within a pH range of 1.0 to 7.2–7.5.

5.2 Medium Additives

Enzymes such as pepsin (at pH <4.0) or pancreatin (at pH ≥6.8) may be used if the product shows evidence of cross-linking in the gelatin capsule. If the product is a formulation such as *2* or *3* above, evaluate the use of surfactants and possible interaction with enzymes.

5.3 Apparatus

USP *Apparatus 1* or *2*. If the capsule is filled with low-specific-gravity liquid and has a tendency to float, sinkers should be used. If the product is a formulation such as *2* or *4* above, consider *Apparatus 3* as an option.

5.4 Agitation Speed

Agitation speed typically is 50–100 rpm for baskets and 50–75 rpm for paddles. Higher speeds should be justified.

5.5 Time Points

A dissolution profile should be established during the development phase to identify when the rate of active ingredient release has leveled off.

6. CRITICAL QUALITY ATTRIBUTES

6.1 Shell Composition

Knowledge of the composition of the shell (polymer, plasticizers, water content, etc.), as discussed at the beginning of this chapter, is an important aspect and helps determine critical quality attributes. Other important aspects of understanding the critical quality attributes are the composition and properties of the gel mass and finished shell. In its simplest form, the shell of a capsule is prepared from a molten gel mass, which in the case of hardgels comprises gelatin or other polymers and water and in the case of softgels the shell is comprised of gelatin and a plasticizer dissolved in an aqueous vehicle. The ratio of polymer to plasticizer varies depending on the desired performance traits of the shell, the size of the shell, and the composition of the fill material. Other minor components added to the gel mass may include colorants, flavors, stabilizers, buffers, and opacifiers. The physical characteristics and quality of these minor components are central to the design of high-quality and robust formulations. The composition of hardgels usually does not vary with capsule size.

6.1.1 GELATIN

Gelatin capsule manufacturing process: For hardgels, there are two separate manufacturing steps: (1) manufacturing the empty shells and (2) the filling process. Capsule shells are produced, packaged, and shipped to the dosage form manufacturers. Capsules are then filled and sealed. Most modern capsule-filling machines are designed to allow accurate filling of powders, granules, pellets, tablets, and combinations of these and many can be modified to allow hot or cold liquid to be filled into hardgels. An essential part of a liquid-filling operation is the ability to effectively seal the capsule. This sealing process is a critical process parameter, and detailed knowledge of all the aspects of this process is important.

For softgels, the formation of the capsule, the filling process (encapsulation process), and sealing of the capsules occur simultaneously. During the encapsulation process, it is important to monitor the shell thickness, seam quality, capsule weight, and the fill weight using statistical process controls.

Chemistry of gelatin: Understanding the chemical nature of gelatin is another important component of the critical attributes of the formulation. Gelatin is graded primarily on the strength of the gel. Depending on the process and the tissue source, noticeable differences in strength are apparent among suppliers and even between lots from the same supplier. Consequently, controlling the strength of the gelatin from batch to batch, measured as Bloom strength, is key to obtaining a consistently performing product. Bloom strength is a measure of the strength of the gel prepared at a set concentration of gelatin in water under controlled conditions and is a function of the molecular weight of the gelatin, the concentration of the gelatin in the gel, and the pH of the gel. It is a measure of the resultant gel's resistance to compression and is reported in Bloom-grams or grams. Bloom strength increases when the gelatin concentration in the gel increases, when the average molecular weight of the gelatin increases, and when the pH of the gel approaches neutrality (from either direction). In addition, as Bloom strength increases, the cost of gelatin increases and the rate of gel dissolution decreases. Bloom strength also has an effect on the clarity and color of liquid-filled capsules. For gelatin with higher Bloom strength, less gelatin is needed to produce a suitable shell,

which results in a clearer shell and a reduced need for colorants and dyes in order to produce the desired hue. Although gelatin can be purchased with Bloom strengths ranging from 50 to 300, most gelatins used in the manufacture of liquid-filled capsules have a Bloom strength of about 150–200 for softgels and 220–280 for hardgels. Gelatin manufacturers commonly blend different sublots of gelatin to meet Bloom requirements. These important characteristics of gelatin must be taken into account when characterizing the product under development.

6.1.2 CHEMISTRY OF OTHER POLYMERS

Suitable consideration should be given to the other polymers used to manufacture capsule shells. Iota and kappa carrageenans, modified corn, potato, and pea starches, and modified celluloses, together with plasticizers are also used for the preparation of capsule shells. Carrageenans are polysaccharides extracted from sea weeds. They possess the required gelling characteristics similar to gelatin. However, kappa carrageenan produces a brittle gel, and iota carrageenan a soft and elastic gel. Carrageenans are usually combined with modified starches and plasticizers to form the capsule shell. Modified celluloses, such as hypromellose (HPMC), are also used for the manufacturing of hardgels. Compared to gelatin shell, the shell prepared using the polymers mentioned above will have some advantages, such as non-crosslinking, being able to handle wider pH ranges, and tolerance of high fill temperature.

6.1.3 PROCESSING AIDS

Processing aids include gelatin ribbon lubricants in the softgel manufacturing process. Commonly used ribbon lubricants include mineral oil and medium chain triglyceride. Since ribbon lubricants are applied to both the inner and outer surfaces of the capsule shell, the evaluation of their potential adverse effects is focused on the capsule itself and primarily on gelatin cross-linkage.

6.2 Stability and Storage Conditions

Knowledge of the stability and reactivity of gelatin or other polymers is essential to anticipate their influence on final product quality. At room temperature, the capsule shells as supplied are relatively effective in protecting the fill from oxygen and its effects, and when an opacifier such as titanium dioxide is added to the shell, it can prevent photodegradation of the fill. In addition, the low water activity of the shell does not promote microbial growth. For bacteria, yeasts, and molds to grow a higher water activity of at least 80 aw is required; the water activity of capsule shells typically is less than 0.40 aw.

Storage conditions of the final product range from refrigeration to standard room temperature. Products should be stored according to the label directions. Brief excursions outside of these conditions should be evaluated to determine their influence on the final product quality. Storage conditions and duration for the empty capsule shells should also be considered.

6.3 Formulation Development and Manufacturing for Liquid-Filled Capsules

It is important to understand the properties of the active ingredient(s) during formulation development.

Formulations developed using liquid-filling technology may be applicable when the active ingredients (1) display poor solubility in aqueous systems, (2) short half-life requiring frequent dosing, (3) low melting point, (4) low dose/high potency where containment is important, (5) requirement for taste or odor masking and (6) critical chemical or physical stability as in the case of highly hygroscopic active ingredients. Other factors to consider during formulation development include:

- Compatibility and stability of excipients and active ingredient(s) over time
- Temperature-dependent solubility of the active ingredient(s) in the lipid
- Aging/polymorphic characteristics of the lipid
- Adequate characterization of the saturation/supersaturation status of the active ingredient(s) in the lipid formulation in order to avoid precipitation of the active ingredient(s). Optimally, saturation status should be determined when the formulation is at equilibrium with the shell.
- Stability in solution under stress conditions
- Aldehyde formation and degradation of active ingredient(s)
- Influence, if any, of the rate of cooling on the structure of certain excipients, which may modify the release characteristics of the active ingredient(s)

Important factors to consider during a liquid-filling operation are temperature and viscosity of the fill material and, in the case of a dispersion or suspension, the particle size of the dispersed active ingredient(s). In principle, any excipient found to be compatible with the shell can be used, but in a manufacturing environment the viscosity of the fill material is important. Excipients that are solid at room temperature but melt at temperatures up to 70° may be suitable (depending on the shell polymer) for formulating active ingredient(s) provided those excipients yield the desired in vivo performance.

Both shell and fill excipients should be controlled for levels of known cross-linking agents such as formaldehyde and reducing sugars.

Imperfections in the shell and/or in the seam may affect dissolution. They may also give rise to leaking of the capsules contents.

The appropriate in-process controls should be in place to monitor and reduce the lot-to-lot variability.

⟨1097⟩ BULK POWDER SAMPLING PROCEDURES

INTRODUCTION

The goals of this chapter are to provide guidance on bulk powder sampling procedures, identify important bulk powder sampling concepts, and collect a knowledge base of useful practices and considerations that can lead to the ideal physical sampling of bulk powder materials. The terminology used here is well established in the field of material sampling (see *Appendix 3*, for instance reference *7*). Sampling is undertaken as part of an estimation process. The parameter of primary interest here is the mean level of some analyte in the bulk powder as a whole.

The purpose of a sampling plan is to obtain a representative sample of a population so that reliable inferences about the population sampled can be drawn to a certain level or degree of confidence. Acquiring a representative sample from a lot is critical because without a representative sample all further analyses and data interpretations about the lot are in doubt. An ideal sampling process is a process in which every particle or at least every equal-size portion of the population has an equal probability of being chosen in the sample. In addition, sampling procedures should be reproducible, i.e., if the sampling protocol were repeated, a high probability should exist of obtaining similar results. Also, the integrity of the sample should be preserved during and after sampling. The details of how to sample depend on a variety of factors. For example, criteria for sampling to evaluate particle segregation may differ from criteria for evaluating moisture content or identification.

Because of the propensity of a powder to segregate, heterogeneous powder systems can make it difficult to obtain an ideal sample. Thus, to extract representative samples requires careful development of a sampling plan that accounts for and mitigates the segregation tendencies of a particular powder system. Developing a general guidance for bulk powder sampling is challenging because every situation is different, and therefore different approaches must be used to deal with each situation. Thus, the goal of this general information chapter is to outline recommended steps for developing a sampling scheme or plan for a particular system that is consistent with good sampling practices.

The primary difficulty in acquiring a representative sample is that the size of the sample for measurement, typically a few milligrams to grams, must be withdrawn from a large population on the order of hundreds to thousands of kilograms. The few milligrams analyzed in a laboratory must be taken from a large population of particles in a warehouse in such a manner that the measurement sample is representative of all the particles in the lot. Any bias or error in the sampling process will cause all future inferences to be in error. Over the years methods have been developed and refined to attempt to ensure that the measurement sample is representative of the whole population. A typical strategy is shown in *Figure 1*. The strategy is to sample in stages, starting with the initial gross or primary sample withdrawn directly from the received containers. In the laboratory, the gross sample must be reduced in size until it is the appropriate size for measurement. This should be done in a manner that minimizes the introduction of sampling errors. The key to reducing the sampling error is to ensure that every particle of the population has an equal probability of being included in the sample. However, because of segregation or the nonrandom nature of powders, many obstacles can cause bias and contribute to sampling errors. Following the flow chart in *Figure 1* and the steps outlined in subsequent discussions will help to minimize sampling errors.

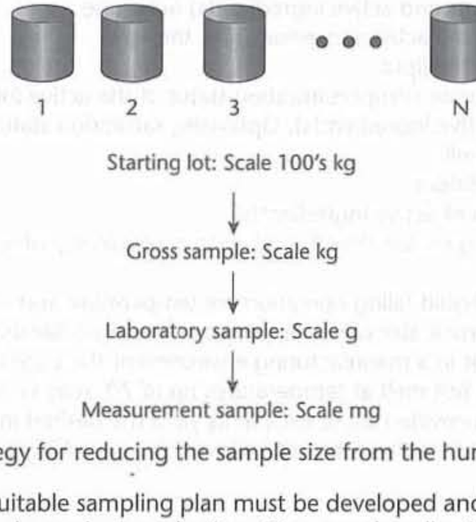


Figure 1. Overall sampling strategy for reducing the sample size from the hundreds of kg scale to the mg scale.

To acquire a representative sample, a suitable sampling plan must be developed and implemented. A good sampling plan includes: (1) population determination and sample size selection, (2) a sample collection procedure and a method for sample

size reduction, and (3) summary calculations that demonstrate that the sampling plan will yield samples that accurately characterize the population to within a stated level of acceptance. In addition, an infrastructure is needed to maintain the integrity of the samples and sampled materials.

This chapter begins with a brief introduction to sampling theory and terminology. The technical content of the chapter requires a basic scientific understanding of physical particle characteristics (e.g., mass, density, shape, and size) and statistics (e.g., acceptance sampling and binomial distribution).

SAMPLING THEORY AND TERMINOLOGY

Fundamental Sample Size (Sample Mass)

Sample size is considered from two perspectives: (1) the mass of the sample intended to represent the entire population, sometimes termed the composite sample, and (2) the number of samples taken with a mass sufficient to independently evaluate, compare, or provide confidence to ensure the reproducibility of the composite or the uniformity of the population. The key to obtaining an ideal sample is to understand and account for the degree of heterogeneity of the characteristic being evaluated in the system under study. For example, heterogeneity of a particle system arises from two sources: the intrinsic, constitutive, or compositional heterogeneity and the spatial distribution heterogeneity. The intrinsic heterogeneity of the powder system reflects the fundamental differences in the individual particles. Statistical heterogeneity (differences between individuals), or variance, is expected to maintain assumed properties. For a normal population the general expression for a statistical sample size suggests that the number of independent samples is proportional to the square of the normal quantile at the desired confidence level (Z) and the population variance (σ^2) and is inversely proportional to the square of the minimum detectable difference required (δ), as shown in equation 1:

$$n \propto \frac{Z^2\sigma^2}{\delta^2} \quad (1)$$

In order to apply the normal theory sample size equation to sample mass with a discrete number of particles, consideration for material characteristics is needed. For a heterogeneous bulk material, such as a bulk powder, the sample mass required to ensure adequate representation of the intrinsic or fundamental population heterogeneity or variation is determined by the size, shape, and density of the particles. The total sampling error (TSE) measures the difference between the analyte concentration estimated in the sample (a_{sample}) and the mean analyte concentration in the lot (a_{lot}) relative to the mean analyte concentration in the lot (a_{lot}), as shown in equation 2:

$$TSE = \frac{a_{sample} - a_{lot}}{a_{lot}} \quad (2)$$

When ideal sampling is employed, the TSE is reduced to a fundamental sampling error, limited only by the intrinsic heterogeneity of the material. The relative variance of the fundamental sampling error ($S_{fse}{}^2$) has been empirically estimated in particle size applications by characterizing the critical particle mass, heterogeneity, size (diameter), shape, density, and weights of the material. Empirical estimates require a thorough and complete knowledge of the material and process. Established material characterization and methods are critical aspects of avoiding unacceptable estimates. As shown in equation 3:

$$S_{fse}{}^2 \propto f_{shape}\, g_{CF}\, c_{max}\, l d_{max}{}^3 \left(\frac{1}{m_{sample}} - \frac{1}{m_{lot}} \right) \quad (3)$$

where f_{shape} is a measure of cubicity or shape factor of the analyte particles; g_{CF}, the granulometric factor, is an empirical correction factor of differences in particle size; c_{max} is the compositional maximum heterogeneity and is calculated as if the material consists of the analyte particles and everything else; l, the liberation factor, is an empirical factor representing the proportion of critical content particles separated from the non-analyte containing particles of the lot; d_{max} is the particle diameter [e.g., the maximum diameter or the diameter (cm) of the size of the opening of a screen retaining 5% by weight of the lot to be sampled]; m_{sample} is the mass of the sample; and m_{lot} is the mass of the lot being sampled. [NOTE—A liberation factor is needed when the analyte does not appear as separate particles. A high liberation value (1.0) suggests heterogeneity of particles. A low liberation value (0.05) suggests very homogeneous particles. See *Appendix 1* for examples of potential applications of equation 3 in the estimation of the fundamental sample mass needed to account for constitutional heterogeneity of the powder mixture.] Use of equation 3 requires prior estimates of f_{shape}, g_{CF}, c_{max}, l, and d_{max}.

Segregation Error

Distribution heterogeneity is the difference between samples or groups of particles spatially or temporally. For example, small particles are located preferentially in the lower portion of a powder bed. This type of situation can arise as a result of

General Chapters

powder bed segregation and is common in some particle systems with a broad particle size distribution. In other words, smaller particles may not be randomly distributed throughout the lot. This spatial heterogeneity introduces variation in the sample and is a source of variation that contributes to the total variation. Together, fundamental and segregation error give rise to sampling error, which dictates how variable the samples will be, how large the sample size and numbers of samples should be (e.g., 10 containers, sampled at top and bottom, with sample sizes of 50 g each), and how hard it will be to obtain a representative sample.

Minimizing the effects of segregation error during lot material characterization while still ensuring a representative sample mass requires collecting many small samples that average out the variation of the segregation error. This assumes one is interested in estimating the overall average, not characterizing lot heterogeneity. Segregation error is difficult to control because segregation may be the result of changes in particle size, shape, and density, as well as inputs into the determination of sample mass. Minimizing the effects of segregation error when reducing the primary sample size requires adequate physical mixing or randomization of the primary samples before analysis, thus providing equal selection probability.

Total Sampling Method Error

Intrinsic or compositional heterogeneity is a function of the powder system and represents the true characteristics of the material (e.g., equation 3). Thus, intrinsic heterogeneity is often the minimal variance a system can have. The difference between the true state of the system and what is actually measured when ideal sampling is employed is called the fundamental error (equation 2). The relative variance of TSE (S^2_{Total}) is represented in equation 4 as the sum of the relative variances of all error components:

$$S^2_{total} = S^2_{fundamental} + S^2_{segregation} + S^2_{extraction} + S^2_{delimitation} + S^2_{preparation} + S^2_{trends,\ shifts} + S^2_{cycles} + S^2_{analytical\ method} \quad (4)$$

The S^2_{Total} can be reduced by employing ideal sampling. Ideal sampling will limit or adjust for the effects of error contributed by particle segregation, extraction error created by the sampling device, delimitation error created by not considering the three-dimensional nature of the bulk material, and sample handling errors such as product degradation. The total variation is the sum of these sources of error, illustrated in equation 4 as independent, additive components. To the end of reducing these errors, an important goal of material characterization by sampling is the determination of the relevant errors within the bulk sample. Knowing the source of the error helps determine how to best minimize these errors.

Fundamental error arises from the intrinsic heterogeneity of particles within a sample of the material population. Reducing fundamental error requires changing the intrinsic characteristics of the material, such as reducing the particle size by milling or grinding. Segregation error is the spatial distributional difference of particles across the population. This type of error can be minimized by mixing or randomization of the particles being selected. Segregation error is affected by the characteristics of fundamental error. Additionally, for the determination of both fundamental and segregation error, it is assumed that mechanical sampling is carried out correctly and is not invasive, i.e., that mechanical sampling does not alter the characteristics being measured and provides a true representation. In instances where sampling of the bulk material does not provide unbiased representation or is so invasive that it alters material characteristics, then, in order to obtain noninvasive, unbiased samples, operators may need to change sampling from a bulk form to a stream form of processing, either upstream or downstream from the sample point (see *Appendix 2*). The mechanical sampler may need to mix the sample sufficiently to facilitate random sampling with equal probability of selection in order to obtain an adequate representation of the entire bulk lot. The process may also require mixing or sampling from a location in the process that will provide a random sample from material that is susceptible to segregation.

Extraction, delimitation, and handling errors occur as a result of the mechanical sampler and sample handling prior to analysis, which also are affected by fundamental error. Trends, shifts, and cycles are temporal sources of error that affect total error. The analytical error of the method of analysis contributes to the overall error of the reported result. In addition to obtaining representative subsamples from the bulk material, the method must also obtain a representative subsample from the particulate laboratory sample before analysis.

Sampling Strategy

A typical sampling strategy consists of two basic steps: (1) the primary or gross sample, followed by (2) the secondary sample, which reduces the primary sample to a size that is suitable for laboratory measurement. In short, the goal is to select from the lot a quantity of material suitable for measurement without significantly changing the attribute for which one is sampling. In parallel with the sample size reduction, sample size calculations must be done in such a way that the sampling strategy has sufficient statistical power to determine whether the attributes of interest lie within the specification ranges with a reasonable degree of certainty. Each step must be done correctly, or the sampling strategy as a whole will not provide a sample that is representative of the original population.

To successfully withdraw a sample from a bulk container that is representative of the population, one needs to have an idea of the population's heterogeneity, i.e., how segregated or stratified the system is. Knowing what factors can accentuate segregation and knowing the patterns of segregation that are likely will help one to account for segregation in a powder bed and to take better samples. Many factors can affect the degree of powder bed segregation. For segregation to occur, sufficient energy

needs to be put into the powder bed to induce motion between particles. When a sufficient amount of energy is supplied, segregation can occur via three modes: percolation (in the powder bed), rolling (on the free surfaces of a powder bed), and free flight (when the powder bed is fluidized). These modes are illustrated in *Figure 2*.

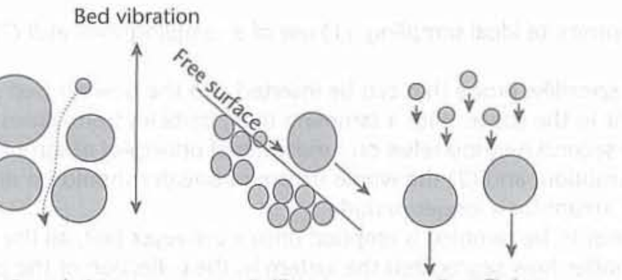


Figure 2. Illustration of the three modes of particle segregation: percolation, rolling, and free flight.

Within the powder bed, segregation can occur by means of percolation, also called sifting segregation, as well as through the movement of coarse particles to the top via vibration. During sifting segregation, smaller particles acting under the influence of gravity can more easily migrate downward into the void spaces between larger particles when the particle bed is perturbed. The net effect of these movements is that the smaller particles percolate down into the powder bed, resulting in the top of the powder bed having a higher proportion of larger particles. A common example of sifting segregation is unpopped corn kernels that are found at the bottom of a bag of popped popcorn.

For free surfaces, rolling segregation can occur any time that particles can roll down a free surface. In other words, segregation can occur on any non-level surface that allows the relative movement of particles. When particles roll down these free surfaces, larger particles tend to tumble farther down the surface than the smaller particles (see *Figure 3*). For example, if a conical heap or pile is formed in the middle of a hopper during loading, larger particles are more likely to roll farther down the heap, toward the outer edge of the hopper. This creates a situation in which the smaller particles tend to be in the center of the hopper, and the larger particles accumulate toward the outer wall of the hopper. The formation of these free surfaces can be a major factor in segregation.

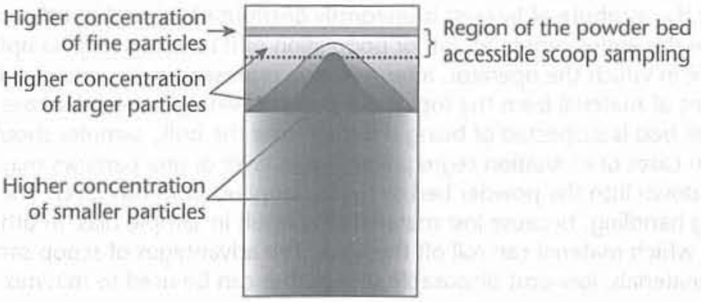


Figure 3. Example of extensive powder segregation within a drum.

When powder beds are fluidized, a large amount of air is incorporated into the powder bed and, when this air is moving, the air velocity may exceed the terminal velocity of the smaller particles. When this happens, the fine particles are suspended in the air stream while the coarse particles settle out. The fine particles eventually settle on top of the powder bed, forming a top layer that has a higher concentration of fine particles. This type of segregation, sometimes called elutriation segregation, can occur when a powder is discharged from a hopper, or is poured into the top of a hopper, and a large volume of air is displaced.

In summary, for a highly segregating system, the powder bed could have a particle distribution similar to that shown in *Figure 3*, where, as a result of elutriation segregation, a layer of fine particles on the top overlies larger particles deposited by percolation segregation, and a radial distribution of larger particles appears toward the outer wall as a result of rolling segregation.

In general, the primary factors that affect segregation are particle size and size distribution, density, and shape and shape distribution. Of secondary importance are surface roughness, surface coefficient of friction, moisture content, and container shape and design. Particle size is the most important single factor, and subtle differences in particle size can cause measurable segregation. If the attribute of interest is associated with particle size, then this attribute will segregate along with the different particle sizes. For example, if a manufacturer makes a granulation in which the larger particles contain more drug than the smaller particles, then drug content can be very prone to segregation—i.e., drug content will show segregation patterns similar to those associated with particle size segregation.

Segregation can notably increase sampling error because it decreases the probability that certain particle types will be in the sample. In addition, the powder bed may already be segregated when material is received, and poor sample handling can also

cause segregation. To avoid further segregation during sample handling, the operator should avoid situations that promote segregation, such as the following: pouring where the powder forms a sloping surface, pouring into the core of a hopper, vibrations, shaking, and stirring (unless done to promote mixing). In addition, the use of mass flow hoppers reduces segregation.

Two basic strategies help promote ideal sampling: (1) use of a sampling thief and (2) sampling from a moving powder stream.

A sampling thief is a long spearlike probe that can be inserted into the powder bed and, once inserted, can collect powder samples from points adjacent to the spear. With a sampling thief, particles from almost any point in the powder bed can be included in the sample. The second method relies on fundamental principles of sampling, namely that (1) a powder should always be sampled when in motion, and (2) the whole stream of powder should be sampled for many short periods rather than sampling a part of the stream for a longer period.

For example, if the container to be sampled is emptied onto a conveyer belt, all the material will pass by a single point that can be sampled. Thus, no matter how segregated the system is, the collection of the powder at random time points ensures that every particle has an equal probability of being included in the sample. The second fundamental principle accounts for material segregation on the conveyer belt: by collecting the entire stream, one gets a cross section of all the particles, no matter how much segregation occurs on the conveyer belt.

Many methods are available for obtaining a sample from a powder system. Unfortunately, many of these methods involve setting the powder bed in motion or performing in-process sampling. Because of concerns about cross-contamination and containment of potentially toxic materials, most of these methods are impractical for the bulk sampling required for compliance with current Good Manufacturing Practices (cGMPs). Hence, most of the sampling done in the pharmaceutical industry is static sampling, done by either (1) scoop or grab sampling or (2) stratified sampling, typically employing a sampling thief. The choice of method is dictated by the distribution of the attribute being sampled in the container, as discussed below.

GENERAL SAMPLE COLLECTION: CONSIDERATIONS AND TOOLS

Types of Systems and General Considerations

HOMOGENEOUS SYSTEMS

For powder systems where the attribute of interest is uniformly distributed throughout the container—so that any sample is an unbiased representation of the entire container, lot, or population of interest—scoop sampling is adequate. Scoop sampling is a straightforward procedure in which the operator, after selecting representative containers for sampling, opens a container, scoops out a sufficient amount of material from the top of the powder bed, and then seals the container. If a thin layer of material on top of the powder bed is suspected of being different from the bulk, samples should be taken from a point below this top layer. For example, in cases of elutriation segregation, a thin layer of fine particles may lie on top of the powder bed, and the operator should dig down into the powder bed to avoid sampling from this layer. The scoop should be large enough that no material is lost during handling, because lost material may result in sample bias. In other words, one should avoid the use of a heaping scoop from which material can roll off the sides. The advantages of scoop sampling are convenience and cost, and, for highly potent materials, low-cost disposable scoops that can be used to minimize cross-contamination.

HETEROGENEOUS SYSTEMS

If the attribute of interest is spatially distributed in a heterogeneous manner throughout the sample, then scoop sampling is prone to potentially significant errors. Scoop sampling is a non-probabilistic method because only the most accessible fraction of the container is sampled. Obviously, only the material in the top layer can be reached with a scoop. For example, a sample from the top outer edge of the drum shown in *Figure 3* could be biased because, in this example, the larger particles are preferentially distributed toward the top and outer edges of the drum. Hence the smaller particles have a lower probability of appearing in the sample. As a result, the smaller particles will be underrepresented in the sample, and any analysis of particle size will not reflect the true particle size distribution of the original population.

For heterogeneous systems, the initial primary sample is the most difficult to obtain. Use of a sampling thief, sometimes called a grain probe or sampling spear, is needed. The advantage of a sampling thief is that much more of the powder bed is accessible because the sampling thief can sample from different points in the powder bed, thus helping to reduce sampling bias. Many types of sampling thieves are available, including: (1) the concentric sleeve with slotted compartments, (2) the concentric sleeve with grooves, sometimes called the open-handled probe, (3) the end sampler, and (4) the core sampler. Each type has its own unique operating procedures, as described below.

The concentric sleeve with slotted compartments is probably the most popular type of sampling thief used in the pharmaceutical industry. This type consists of two concentric tubes or cylinders in which the inner tube is divided into compartments. This design makes it possible to detect differences in the attribute of interest across the depth of the container. To collect a sample, the operator closes the compartments and inserts the sampling thief into the powder bed with the collection zone openings facing upward. The handle is turned to open the sample zones, then the handle is moved up and down with two

quick short strokes to help fill the compartments. The sampling thief is then closed and removed from the powder bed. The operator should visually inspect the powder bed through its depth before emptying the sampling thief. The powder from the individual compartments can be combined on a clean surface or in a collection container. In certain situations the material from each compartment may be analyzed separately, that is, without mixing.

In the concentric sleeve with grooves (open-handled probe), the inner tube is not divided into compartments. The probe is first inserted into the powder bed with the groove open, the outer sleeve is rotated to close, and the sampling thief is then withdrawn from the powder bed. The probe's contents are emptied from the handle end by holding the probe upright and letting the sample slide out from the handle, a method more convenient than the one using the thief with slotted compartments. However, this type of thief makes it more difficult to perform visual inspection to examine for material inconsistencies according to depth.

An end sampler probe, often used to sample slurries, has a single entry zone at the bottom of the sampling thief. Frequently the end sampling zone is larger than the rest of the sampling thief. This feature is a disadvantage because the larger the probe, the more it perturbs the powder bed, possibly resulting in the introduction of sampling bias.

Core samplers have a hollow outer cylinder with a tapered outer wall on the open end. This probe is inserted into the powder bed, and the intrinsic cohesion of the particles keeps them from flowing out when the probe is withdrawn. The contents of the cylinder are then emptied into a clear container.

GENERAL CONSIDERATIONS

The most reliable and reproducible results in powder size measurements are obtained when the particle size ranges from 2 to 10 µm; otherwise, the powder is too cohesive and does not flow properly into the sampling thief. In addition, particles larger than about one-third the width of the slot give poor results. Samples should be taken from several sites throughout the container. The probe should be long enough to penetrate at least three-quarters of the depth of the powder bed, ensuring that material from all depths can be captured in the sample. The choice of sites should be dictated by an understanding (often subjective) of the powder bed's degree of heterogeneity, which may have been caused by handling or movement during transport. Sampling plans can call for the insertion of the probe either at random locations and random angles or at predetermined locations and angles. For example, the plan may call for the probe to be inserted at the center and at two locations near the edges. Also, many operators recommend that probes always be inserted at a 10° angle from vertical, which increases the range of locations sampled.

Some of the disadvantages of sampling thieves include the labor-intensive nature of the procedure. The probe must be physically inserted into the powder bed, often multiple times; the contents of the probe must be emptied; and then the probe must be thoroughly cleaned. For settled powder beds, the sampling probe can be difficult to insert. In addition, the sampling probe can introduce errors as a result of the following: fine particles can lodge between the inner and outer tubes; particles can fracture; fine particles can compact and not flow well into the sampling compartments; segregation can occur during flow into the sampling zone; and the act of inserting the probe can disrupt the powder bed by dragging powder from the top layers of the bed down through the bed.

Representative Lot Sampling

Statistically-based sampling plans are based on statistical principles and depend on the population's spatial heterogeneity and intrinsic variability. Statistically-based plans are efficient and allow the collection of a sufficient number of samples to yield the desired degree of certainty without collecting too many or too few samples for the test method, scale, product variation, risk requirements, and tolerance for a stated product's quality level or specification. The commonly used $\sqrt{N} + 1$ sampling plan given in *Table 1* is not a statistically based sampling plan and may result in collection of too few samples for small populations and too many samples for large populations. The use of statistically-based sampling plans is advantageous because it facilitates risk management. However, in cases where prior knowledge of the population to be sampled is insufficient, a nonstatistical sampling plan such as that given in *Table 1* can be considered.

Figure 4 illustrates the sample size selection scheme paths. The first choice is whether to use a statistical or nonstatistical sampling plan. Statistical plans are preferred when a variable attribute like particle size or drug content is being determined. General sampling approaches are outlined in *USP* general information chapter *Analytical Data—Interpretation and Treatment* 〈1010〉. Statistically-based lot acceptance sampling plans require a valid rationale with known quality levels for the determination of product lot characteristics. As noted, the application of statistical sampling plans, including lot acceptance sampling plans, requires specific and thorough knowledge of the material being sampled. Reference statistical sampling plans state the rationale for sampling as part of the sampling scheme. Manufacturers who use a statistically-based lot acceptance sampling method should refer to an appropriate standard such as ANSI/ASQ Z1.9-2003 for bulk materials or ANSI/ASQ Z1.4-2003 for multiunit or discrete populations. These standards are readily available via sources such as the American Society for Quality (http://www.asq.org/) or the American National Standards Institute (http://www.ansi.org/).

General Chapters

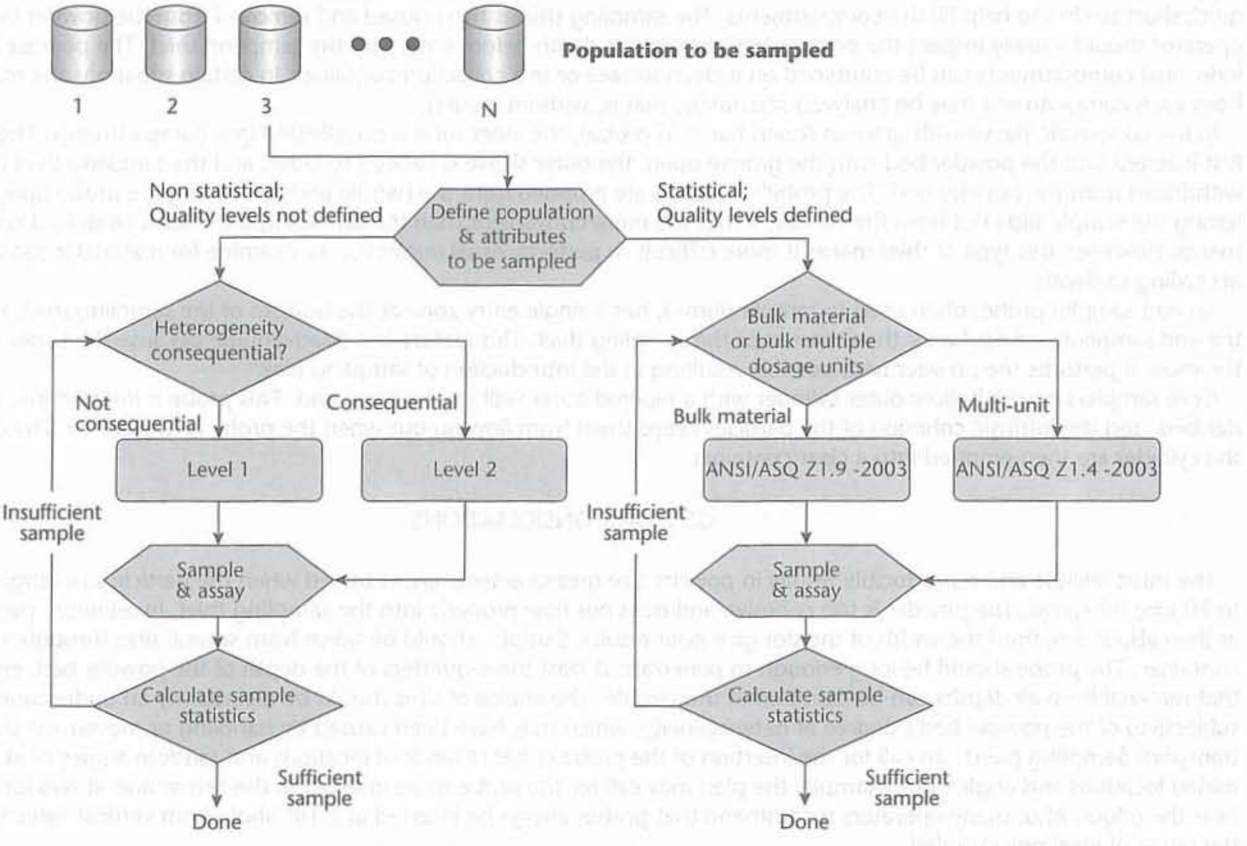


Figure 4. Sample size selection scheme.

If one is developing a nonstatistical sampling plan for which the quality level is not known, *Table 1* gives suggested sample sizes for the number of containers in the lot that should be sampled.

The *Level 1* sampling plan is relevant to materials when heterogeneity does not affect the analysis and the customer seeks to sample more than one container, when the sampling plan can be proportional to the square root of the number of containers received, and when the material comes from a known and trusted source. In such cases, the sample can be withdrawn from any point in the container. For adequately homogenous systems, scoop sampling from the top of the container is suitable.

The *Level 2* sampling plan involves a 50% increase in sample size when compared with *Level 1* and is used when a larger proportion of the number of containers is needed, for example, when a material's heterogeneity is suspected of being consequential and acceptance sampling quality levels are not defined, or when the material comes from a less trusted source. Depending on the material's degree of heterogeneity, a sampling thief may be used. However, if the degree of heterogeneity will not significantly affect the results for the attribute being sampled, then scoop sampling from the top of the drum may still be suitable.

Table 1 shows the number of containers, n, to be sampled for a lot segregated into N containers. Note that the value of n from the formula is rounded at 0.5 up to the next higher integer. For example, if N = 6: for *Level 1*, n = $\sqrt{6} + 1 = 3.45$ rounds to n = 3; for *Level 2*, n = $1.5 \times \sqrt{6} = 3.67$, which rounds to n = 4.

Table 1

N (Number of Containers Comprising Lot)	**n (Sample Size)**	
	Level 1	**Level 2**
$N \leq 3$	All	All
$N \geq 4$	$\sqrt{N}+1$	$1.5 \times \sqrt{N}$

These initial decisions, as illustrated in *Figure 4*, are often difficult and sometimes must be made without sufficient information. If there is uncertainty about which method or level is appropriate, sometimes a quick, small-scale informal test of the system may help determine the best way to proceed. In addition, for some systems and attributes, the *Level 1* and *Level 2* sampling plans may result in oversampling. For example, when one is sampling for identification from the same lot, the suggested levels may result in collecting more samples than are statistically needed; in such cases, the statistically-based sampling plans referenced in *Figure 4* can be used.

Sample Collection

Acquiring a representative sample from a lot of bulk powder is a difficult procedure that requires special consideration, and the basic procedures for acquiring a representative sample are discussed below. Note that every situation requires techniques that are appropriate for the given population to be sampled. The methods presented here are applicable to the sampling of static powders stored in midsize bulk containers such as 1-ton super sacks, 50-kg drums, or 50-lb bags. These methods are not necessarily applicable to the sampling of liquids, large storage containers such as train cars or silos, or in-process systems such as blenders or moving conveyer belts. In addition, the procedures described here are most applicable to particles in the size range from approximately ~1 µm to approximately ~1000 µm. Significantly smaller or larger particles require special procedures that are not covered here.

PRIMARY SAMPLE COLLECTION

Lot acceptance samples are generally transferred or delivered in containers. To collect a representative primary or gross sample (see *Figure 1*), the appropriate container or containers must first be selected from the population of N containers; second, a representative sample must be withdrawn from each of the selected containers.

Container Selection

To avoid bias and other sampling errors, the containers to be sampled must be randomly selected. To make a random selection, first number all containers in the lot, then use a random number table (or computer-generated random numbers) to choose from which container or containers to withdraw the samples.

For systems in which containers are grouped together in such a manner that many of the individual containers are not practically accessible (e.g., 50-lb bags stacked and bound in shrink wrap on a pallet), the sampling plan may need to take into account the larger container, in addition to the smaller container, as a sampling unit, in order to ensure a representative sample.

Withdrawing Sample from a Container

CONTAINER TYPES

The three most popular container types are the bag, drum, and super sack. Because bags are generally closed and not resealable, special sampling thieves, sometimes called bag triers, have been designed to puncture the bag. If the system to be sampled is heterogeneous, the samples should be obtained from the bottom, center, and top of the bag; and, depending on how the bags are stacked on the pallet, they should also be sampled from the front and the back. When sampling from bags, particular attention should be paid to the corners, because they can disproportionately trap fine particles. If no bag trier is available, use a knife to cut open the bag for sampling. When sampling from a bag, be sure to clean the external surface sufficiently that the sample is not contaminated and foreign material is not introduced into the bulk material. Once the sample has been taken, place a compatible material over the hole in the bag, then fix this patch with an appropriate adhesive tape. Depending on the heterogeneity of the drum, a scoop or a sampling thief is used. Super sacks are large sack containers that usually have a fill spout on the top and a discharge spout on the bottom. For adequately homogeneous material, scoop sampling is appropriate; but if there is any concern about the heterogeneity of the material, a thief should be used. The large size of super sacks makes the use of a thief more important for representative sampling than in the case of a drum or bag, in order to limit potential delimitation error.

Sample Handling

The samples collected can be either assayed individually or combined; then a subset of the gross sample can be assayed, as depicted in *Figure 1* and described below. Sample increments should be combined on a clean, dry surface or in a suitable container or bag. All containers with which the sample comes into contact should be inert and should not chemically or physically react with the sample. In addition, samples should be accurately labeled and good records kept. A portion should be kept for possible future analysis.

PRIMARY SAMPLE SIZE REDUCTION

As mentioned above, the primary sample typically consists of multiple samples taken from containers and mixed together. To obtain an analysis or measurement sample (*Figure 1*), the gross or primary sample must be reduced to a size appropriate for the analytical method. Gross or primary sample size reduction is an often overlooked aspect of a sampling plan, but it is an important step. The factors that cause segregation in a container can also cause segregation in the primary sample, and any bias in the size reduction method for the primary sample will lead to erroneous results. The advantage of secondary samples is

that the mass has been reduced to a point at which it is much easer to obtain a representative sample because every element in the powder bed is readily accessible. Such accessibility makes it easier to adhere to sampling best practices. Generally speaking, sample measurement takes place under either wet or dry conditions; the choice is dictated by the requirements of the analytical method. For example, the Coulter counter requires that samples be uniformly suspended in an electrolyte, but other methods, like sieving, are typically performed with dry powders.

Before dividing an agglomerated sample, the agglomerates should be broken apart by a suitable technique such as sieving.

Dry Analysis Methods

Many laboratory devices are available for the reduction of the primary sample to an analytical sample. The three most important methods used in the pharmaceutical industry are: (1) scoop sampling, (2) cone and quartering, and (3) the spinning riffler or rotary sample divider (manual method of fractional shoveling); see *Figure 5*.

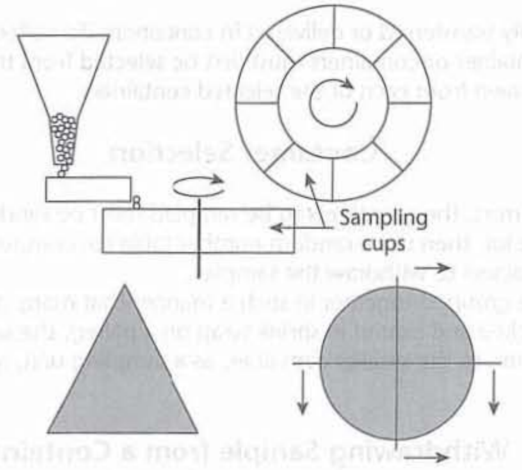


Figure 5. Two procedures for dividing samples. Top: spinning riffler, in which a circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Bottom: cone and quartering. (Cone, left, is flattened and quartered; quarters can be formed into cones and further subdivided.)

SCOOP SAMPLING

Scoop sampling is done as previously described, but generally with a smaller scoop or spatula. Great care must be taken when removing material from the primary sample, because this material could be highly segregated as a result of handling. Scoop sampling is appropriate for homogeneous or cohesive powders. However, if the powder is prone to segregation, scoop sampling can introduce significant errors. Moreover, scoop sampling has several serious disadvantages. First, the method depends on the operator's deciding from which part of the primary sample to scoop the material and what quantity of the sample to extract, which are features that can introduce operator bias. Second, in scoop sampling, operators have a natural tendency to withdraw the sample from the free surface, which is highly prone to segregation and is not representative of the bulk. Third, operators need to avoid creating a heap where rolling segregation can occur, because material could fall off the edges of the spatula or scoop and bias the sample. Ideally, the operator should make some attempt to mix the primary sample before using the scoop, but this too can exacerbate segregation problems and should be done only with great caution.

CONE AND QUARTERING

Cone and quartering is done by pouring the primary sample into a symmetric cone on a flat surface. The cone is then flattened by a flat surface such as a spatula, and is divided into four identical quarters (*Figure 5*). One quarter is taken as the sample. This procedure can be repeated (e.g., quarter-samples can be subdivided into quarters) until the desired sample size is obtained. The theory of this method is that when a symmetric cone is created, all the segregation processes also occur symmetrically around the cone, and hence symmetry is used to mitigate the effect of segregation. In practice, it is very difficult to actually make a symmetric powder cone, and the method becomes very operator-dependent and often unreliable. Differences in how operators form the heap and subdivide it can lead to a lack of precision and significant errors. In addition, if the method is done more than once, errors can propagate each time the cone and quartering is performed. Some experts do not recommend this method.

SPINNING RIFFLER

A spinning riffler (*Figure 5*) includes a series of containers mounted on a circular holder. The circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Once the material has been divided among the different holders, an individual holder can be removed for testing or further sample division. The angular velocity of the circular holders and the amplitude of the vibratory feeder can be controlled to accommodate powders with different flow properties. The holder velocity and feed rate should be adjusted so that the containers fill uniformly and so that a heap does not form on the vibratory feeder. Spinning rifflers are available in different sizes, making possible subdivisions of powders ranging from a few milligrams to hundreds of grams. The only drawbacks of the spinning riffler are the time required to process the sample and clean the device, and the capital expense. Despite these minor disadvantages, the spinning riffler is by far the best method for subdivision of free-flowing powders.

Fractional shoveling is the manual version of the spinning riffler. In this method, scoop samples are taken from the original sample and placed into a sufficient number of aliquots, and then subsequent scoops are taken from the original sample and placed into one of the aliquots in sequential order. This process is repeated until the original samples are gone. Then one of the aliquots is randomly taken as the reduced sample. As is the case with all manual methods, operator error and variability can be significant factors.

Wet Analysis Methods

Wet analysis methods require dispersing the sample in a liquid suitable for analysis, and then withdrawing an aliquot using a syringe or pipet. Effective secondary sampling requires making a stable homogenous suspension (i.e., the sample must be stable from the time of formation of a suspension to the time when the analysis is complete). Some important factors in wet analysis are sample solubility in the dispersion vehicle, aggregation of sample, the use of suspending agents, and deaggregation of primary particles in the dispersion vehicle. Even though a uniform suspension is created, the sample should be homogenized, typically by shaking, immediately before withdrawing the sample with a syringe or pipet. The diameter of the syringe or pipet should be large enough so that particles are not excluded and clogging does not occur. The diameters of the largest particles should not exceed 40% of the syringe or pipet tip diameter. If for practical reasons the amount of material from the primary sample is too large, the sample size should be reduced before a suspension is made. To reduce the sample size, use the methods described above in the *Dry Analysis Methods* section. As a precaution, collect and retain enough sample to repeat all tests a minimum of five times.

APPENDICES

Appendix 1: Subsampling Examples

The examples provided below describe the importance of material particle characterization during the selection of an appropriate sample mass. Four examples are presented. In the first example, similarity in the fundamental or intrinsic material characteristics is assumed. In the second example, the density of the heavy metal analyte being measured is changed. In the third example, the effect of changing the particle size is evaluated. In the fourth example, the adequacy of the fundamental particle characteristics in a formulation needed for a given unit dose or mass is evaluated.

EXAMPLE 1. SAMPLE MASS DETERMINATION

Assuming the lot size is 1 kg, the maximum particle diameter is 1000 µm, and the concentration of the analyte is expected to be 1%, what sample mass of round, equal-sized and -shaped 1000-µm particles with a density of 1 g/cm³ would be needed to estimate the average concentration of the analyte with a percent relative standard deviation (%RSD) of 5%?

Rearranging equation 3, one can estimate the sample mass as shown in equation 5:

$$m_{sample} \approx \frac{1}{\frac{S_{fse}^2}{f_{shape}\, g_{CF}\, C_{max}\, l d_{max}^3} + \frac{1}{m_{lot}}} \quad (5)$$

The compositional maximum heterogeneity (c_{max}) can be estimated by considering the analyte and matrix density (λ_a and λ_m, respectively, and their average λ) and analyte concentration (a_L) (equation 6):

$$C_{max} = \frac{(1-a_L)^2 \lambda_a \lambda_m}{a_L \bar{\lambda}} \quad (6)$$

For low analyte concentrations, the compositional maximum heterogeneity is simplified to equation 7:

$$C_{max} \approx \lambda_a / a_L \qquad (7)$$

For high analyte concentrations, the compositional maximum heterogeneity is simplified to equation 8:

$$c_{max} \approx \lambda_m \left(1 - a_L\right) \qquad (8)$$

The shape factor is approximated by equation 9:

$$f_{shape} \approx Volume / d^3 \qquad (9)$$

Where d is the nominal particle diameter for a sphere, and the shape factor is [(4/3)π/8], or approximately 0.5.

The granulometric factor can be approximated by the typical minimum diameter noted as the 5th percentile size, divided by the typical maximum diameter noted as the 95th percentile size, as shown in equation 10:

$$g_{CF} \approx d_{5\%} / d_{95\%} \qquad (10)$$

Because all particles are the same size, the granulometric factor, g_{CF}, is 1.0. Because the analyte exists in a state liberated from the matrix particles, the liberation factor is also 1.0. The sample mass for a 5% RSD (using equation 5) is then:

$$m_{sample} = \frac{1}{\dfrac{0.05^2}{0.5 \times 1 \times 100 \times 0.1^3} + \dfrac{1}{1000}} = 19.6 \text{ g}$$

A sample mass of 19.6 g will provide a sampling error of approximately 5% RSD. Note that in this example the particle characteristics are simplified to demonstrate that a lot mass of 1000 g contains 2×10^6 particles of 0.5 mg mass. The sample mass of 19.6 g contains approximately 39,216 particles, yielding a 5% RSD, using the binomial distribution where p is the concentration of the analyte (a_L) and n is the number of particles sampled, as shown in equation 11.

$$\text{Binomial RSD} = \sqrt{(1-p)/np} =$$
$$\sqrt{(1-0.01)/39{,}216 \times 0.01} = 0.0498 \approx 0.05 \qquad (11)$$

(See *Table 2* for a summary of calculations.)

Table 2. Summary of Calculations for *Example 1*, Equal-Sized and -Shaped Particles

m_{Lot} (g)	d (cm)	f_{shape}	g_{CF}	c_{max}	a_L	λ_a(g/cm³)	l	m_s (g)
1000	0.1	0.5	1	100.0	0.01	1	1	19.6
Mass per Particle P_s $d^3 f_{shape} \lambda_a$			Particles in 19.6 g $m_s/(P_s/g_{CF})$				Binomial RSD $p = a(_L) = 0.01$ $n = 39{,}216$ (Eq. 11)	
0.005			39,216				0.05	

In determining the required sample mass, it is assumed that the sample is representative of the population. Moreover, when using a single representative sample, it is assumed that the uniformity of the sample mass is consistent with the remaining population. Note that the granulometric and liberation factors allow proportional adjustment of the sample size, depending on the nature of the particles. The inclusion of a liberation factor in the equation allows for particles to exist with a proportion of the analyte residing within every particle or a proportion thereof. The granulometric factor permits adjustment of the sample mass by accounting for the relationship in size between the smallest and largest particles represented in the lot.

This approximation also can be applied to liquid suspensions in which each particle is considered discrete and the sample can be characterized with respect to size, density, mass, and volume.

EXAMPLE 2. HEAVY METAL

In this example, it is assumed that the analyte is the heavy metal lead, with a density of 11.34 g/cm³, with a limit of not more than 5 ppm, where the shapes of the particles are cubes (f_{shape} = 1.0), the particles are approximately 50 µm, and a 5-g sample is taken from screened material (g_{CF} = 0.55). On the basis of equation 3, the %RSD is 17.7%. Using equation 5, one finds that a sample mass of approximately 60 g is needed to achieve a 5% RSD, assuming that a_L is equal to the limit allowed and that the analyte cannot be assumed to be liberated from the material (l = 1.0). (See *Table 3* for a summary of calculations.)

Table 3. Summary of Calculations for *Example 2*, Heavy Metal

m_{Lot} (g)	d (cm)	f_{shape}	g_{CF}	c_{max}	a_L	λ_a (g/cm³)	l	m_S (g)
1000	0.005	1.0	0.55	2.3×10^6	5×10^{-6}	11.34	1.0	58.71

If the sample were assumed to be homogeneous (l = 0.1) with respect to presence of the analyte with all particles, then a sample mass of 6.2 g would be required. Moreover, if the shape of the particles were between round and cubic (f_{shape} = 0.8), then a sample mass of 5 g would be required to complete the analysis.

EXAMPLE 3. SUBSAMPLING

Ideal sampling, as noted earlier, is fundamental to understanding the important role of subsampling. In many instances it is desirable to reduce the sample size in a manner that results in a representative sample and lessens the need to test a large sample mass. In some cases the particle size and compositional heterogeneity can result in an unwieldy sample mass. This may occur with larger-sized particles or when a composite sample of many containers is required. Samples with larger-sized particles may need to be physically reduced.

For example, using *Example 2* above, if the maximum particle size were 1000 µm or 1 mm, then a 997-g sample would be suggested by equation 3. Reducing the particle size by grinding and subsampling to achieve a predetermined sampling %RSD may require subsampling more than once to achieve the desired particle size. For example, the entire sample may be reduced to 100 µm to reduce the %RSD to approximately 3%; then, with ideal sampling, a subsample could be selected and entirely reduced to 50 µm to achieve a 5% RSD. Finally, a 5-g subsample could be correctly taken and tested. If certain particles have a large size with high concentration of the analyte, then samples should be selected to ensure that at least 1, but preferably at least 5–6, particles would be selected with 95% probability or chance of selection.

EXAMPLE 4. MINIMUM UNIT DOSAGE MASS

A formulator would like to know the minimum mass required for a dosage form to ensure with 95% confidence a unit dosage of 1% active drug powder. The drug and the excipient have a similar round shape (f_{shape} = 0.5) and a density of 0.33 g/cm³. The active drug is milled to 1 µm, but the size of excipients can be as large as 200 µm. The value for g_{CF} is taken from equation 10 using the expected range of the excipient that accounts for 95% of the formulation, as 10 µm/200 µm, or g_{CF} = 0.05. The quantity c_{max} from equation 7 is taken as 0.33/0.01. The drug particles are completely liberated from the excipient. The batch size is 100 kg.

A minimum sample mass of approximately 3 mg is needed to ensure with 95% confidence (2 RSDs) that the average drug content is 0.9%–1.1%. The proposed dosage form has an active concentration of 100 µm/10 mg total unit mass. The unit dosage form mass is adequate, but the formulation requires that the mixing process, unit dosage production, bulk sampling device, and lab sample preparation or subsampling from bulk samples result in equal probability of selection of drug particles. Only if these conditions for mixing, production, sampling, and testing are met can it be reliably demonstrated that the unit dosage and test determination acceptance criteria of 1% (0.01 µg/mg) are met. Acceptable outcomes of such testing also indicate that the particle size, shape, and density must be controlled. For example, an increase in the sizes of particles to 500 µm results in a need for a 42-mg sample mass and dose. Assuming a cubic, as opposed to a rounded, particle increases the sample mass to 5 mg, which for a fixed dosage form mass may result in less room for the variation contributed by other characteristics, or in lesser confidence. If the acceptance criteria were changed to 0.95%–1.05%, requiring a 1% RSD, then the minimum sample mass would increase to approximately 70 mg. (See *Table 4* for a summary of calculations.)

Table 4. Summary of Calculations for *Example 4*, Minimum Unit Dose Mass

m_{Lot} (g)	d (cm)	f_{shape}	g_{CF}	c_{max}	a_L	λ_a (g/cm³)	l	m_S (g)
10^5	0.02	0.5	0.05	33	0.01	0.33	1.0	0.00264

Appendix 2: Material Characterization and Sampling

Specific and thorough knowledge of the material's synthesis, composition, and usage is critical to developing a bulk material sampling plan. Material characterization is important because bulk material can exist in many forms throughout the material process flow. As illustrated in *Figure 6*, the type of sampling can vary by process step and ultimately affects the use of the material in the drug product. Appropriate material characterization considers the material process step, the type of sampling, the objective of the process step, and ultimately the drug product.

General Chapters

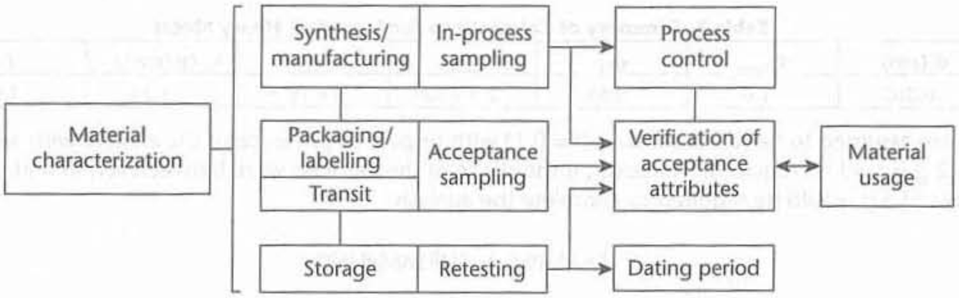


Figure 6. Material process flow.

For example, material can be synthesized or mixed in a large container where sampling may be limited or ideal for the characteristic needed. If the characteristic is important but the sampling conditions are not ideal, perhaps because of the heterogeneity of a powder mixture, then sampling for that characteristic may be more appropriately performed at a different stage upstream or downstream to assess the heterogeneity of the contents and ensure ideal sampling. This is sometimes performed to reduce the sampling dimension. The sampling dimension is reduced when the 3 dimensional bulk container space is sampled in a 1 or 2 dimensional stream sampled over time. Reducing the spatial sampling dimension may result in conditions that will allow for more accurate measurement of the heterogeneity of material while limiting sampling error through ideal sampling.

Acceptance attributes (see *Table 5*) depend on material characterization and process. Acceptance attributes may be applicable throughout the life of the bulk material. Both the number and size of samples require an understanding of the material's variation.

Table 5. Examples of Acceptance Attributes

Acceptance Attributes			
Physical	**Chemical**	**Microbiological**	**Packaging**
Particle size Viscosity Density	Purity pH Identity Strength	Sterility Pyrogens Microbial load	Label accuracy Integrity

Appendix 3: Additional Sources of Information

1. Allen, T, *Particle Size Measurement, 5th ed., Vol. 1*, Powder Technology Series, B. Scarlett and G. Jimbo (Eds.), 1997, London: Chapman & Hall, p. 525.
2. Beebe, KR, RJ Pell, and MB Seasholtz, *Chemometrics: A Practical Guide.* 1998, New York: John Wiley & Sons, p. 348.
3. Brereton, RG, *Chemometrics Data Analysis for the Laboratory and Chemical Plant.* 2003, Chichester, England: John Wiley & Sons, p. 489.
4. Burns, DA, and EW Ciurczak, *Handbook of Near-Infrared Analysis: Practical Spectroscopy, 2nd ed.* 1992, New York: Marcel Dekker.
5. Chowhan, Z, Segregation of particulate solids, Part 1. *Pharm. Technol.*, 1995, 19(May): pp. 56, 58, 60, 62, 64, 66, 68, 70.
6. Chowhan, Z, Segregation of particulate solids, Part 2. *Pharm. Technol.*, 1995, 19(June): pp. 80, 82, 84, 86, 88, 90, 92, 94.
7. Gerlach, RW, and JM Nocerino, *Guidance for Obtaining Representative Laboratory Analytical Subsamples from Particulate Laboratory Samples.* U.S. Environmental Protection Agency EPA/600/R-03/027, November 2003.
8. Jillavenkatesa, A, SJ Dapkunas, and L-SH Lum, *Particle Size Characterization*, Special Publication 960-1, National Institute for Science and Technology, 2001.
9. Kramer, R, ed., *Chemometric Techniques for Quantitative Analysis.* 1998, New York: Marcel Dekker, p. 203.
10. Malinowski, ER, *Factor Analysis in Chemistry, 3rd ed.* 2002, New York: John Wiley and Sons, p. 414.
11. Martens, H, and T Naes, *Multivaritate Calibration.* 1989, Chichester, England: John Wiley & Sons, p. 419.
12. Muzzio, F, et al., Sampling and characterization of pharmaceutical powders and granular blends. *Int. J. Pharm.*, 2003, 250(1): pp. 51–64.
13. Muzzio, FJ, et al., Sampling practices in powder blending. *Int. J. Pharm.*, 1997, 155(2): pp. 153–178.
14. Petersen, L, CK Dahl, and KH Esbensen, Representative mass reduction in sampling—a critical survey of techniques and hardware. *Chemom. Intell. Lab. Syst.*, 2004, 74: pp. 95–114.
15. Petersen, L, P Minkkinen, and KH Esbensen, Representative sampling for reliable data analysis: theory of sampling. *Chemom. Intell. Lab. Syst.*, 2005, 77: pp. 261–277.
16. Sastry, KVS, et al., Solid-solid operation and equipment, in *Perry's Chemical Engineeer's Handbook*, RH Perry, DW Green, and JO Maloney, Eds. 1997, New York: McGraw-Hill, pp. 19-1–19-65.
17. Skoog, DA, and JJ Leary, *Principles of Instrumental Analysis, 4th ed.* 1992, Fort Worth, TX: Saunders College Publishing, p. 700.

General Chapters

18. Smith, PL, *A Primer for Sampling Solids, Liquids, and Gases: Based on the Seven Sampling Errors of Pierre Gy.* In ASA-SIAM Series on Statistics and Applied Probability, RF Gunst, ed. 2001, Philadelphia: Society for Industrial & Applied Mathematics. p. 96.
19. Tatavarti, AS, et al., Assessment of NIR spectroscopy for nondestructive analysis of physical and chemical attributes of sulfamethazine bolus dosage forms. AAPS *PharmSciTech*, 2005, 6(1): pp. E91–E99.
20. Venables, H, and J Wells, Powder sampling. *Drug Dev. Ind. Pharm.*, 2002, 28(2): pp. 107–117.

⟨1102⟩ IMMUNOLOGICAL TEST METHODS—GENERAL CONSIDERATIONS

INTRODUCTION

This general information chapter provides a high-level description of principles for immunological test methods (ITMs) that can be used in specified monograph tests, along with information and approaches to analytical development and validation for ITMs. The scope of this chapter is to provide general information that is applicable to all ITMs. The chapter provides a foundation for specific chapters about different types of ITMs, e.g., *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩ (proposed), and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩. This suite of general information chapters is related to the bioassay general information chapters. Use of ITMs for process monitoring, diagnosis, and evaluation of clinical response, assessment of pharmacokinetics/pharmacodynamics/absorption, distribution, metabolism, and excretion (PK/PD/ADME), and other product characterization (nonrelease testing) is outside the scope of this chapter.

The basis of all ITMs used to measure a quality attribute of a biologic drug substance or drug product is the highly specific noncovalent binding interaction between an antibody and antigen. The antigen typically is an analyte of interest (e.g., protein, carbohydrate, virus, or cell), and the binder is usually an antibody (e.g., monoclonal antibody or polyclonal antiserum). ITMs are applicable to molecules that are either directly antigenic (immunogens) or can be rendered indirectly antigenic (haptens). The measurand in ITM is directly related to a quality attribute of the product under test.

ITMs are valuable because they exhibit high sensitivity and specificity for an analyte in complex matrices. They typically are used for qualitative and quantitative assessment of both an antibody and antigen, but their application also extends to the measurement of hapten, complement, antigen–antibody complexes, and other protein–protein interactions. These properties of ITMs allow their use for assessing identity, potency (strength), purity, impurities, stability, and other quality attributes of biological drug substances and drug products.

ITMs are useful for many applications because they can measure molecules over a wide range of sizes and binding types. In general, antibodies are stable during various chemical modifications that do not have a significant adverse influence on interactions with an antigen. Antibody molecules tend to withstand moderate acidic and alkaline pH changes better than other proteins do. Because of this characteristic, a variety of ITMs with high degrees of sensitivity and specificity are possible. The ability to accelerate contact between an antigen and antibody enables ITM formats that provide rapid or real-time results.

Generally, ITMs have higher precision and shorter turn-around time than do traditional biologically-based (i.e., cell-based and animal) assays. Although in some cases these advantages can support the replacement of a biological assay with an immunoassay, such changes should be approached systematically and with caution. Often it is challenging to prove the equivalence, or comparability, of results from bioassays and immunoassays because the interaction between antigen and antibody may not reflect the functional attributes observed in bioassays.

One major limitation of ITMs compared to physicochemical methods (such as liquid or gas chromatography) is that the latter generally are more precise and can simultaneously identify a set of impurities or unexpected substance(s). Another major limitation is that generally ITMs operate at high molar dilutions at which they are sensitive to disturbances caused by environmental factors in the sample matrix (i.e., matrix effects). Matrix effects can depend on ITM format and are not fully understood. Their specificity, a hallmark of ITMs, is sometimes compromised by structural or sequence similarities between the analyte and a closely related molecular impurity (cross-reactivity).

Most ITMs reflect physical interaction (binding) between an antigen and antibody and not the analyte's functional properties. Therefore, analysts must pay attention in the selection and execution of ITM format. Cell-based ITMs that can provide functional information about the analyte are beyond the scope of this chapter.

GENERAL CHARACTERISTICS OF ITMs

ITMs are based on the principle of specific, noncovalent, and reversible interactions between an antigen and antibody. In general, the primary antigen–antibody reaction is brought about by complementarity, which creates macromolecular specificity. This noncovalent interaction determines the degree of intrinsic affinity. Intrinsic affinity contributes to functional and/or relative affinity that depends on factors like reaction phase and valency, which in turn determines the degree of reversibility of an

interaction. A better understanding of factors that affect antigen–antibody interactions provides the rationale for the development of a suitable ITM format (e.g., solid or liquid phase, competitive or noncompetitive binding, etc.).

A defining characteristic of ITMs is that they employ an antigen (or hapten) and antibody. In addition, ITMs may contain companion molecules such as complement components. The components of ITMs are defined as follows:

- **Antigens**—Comprise a wide range of molecules that are capable of binding to the antibody in a specific interaction. Generally, part(s) of an antigen (the immunogenic epitope[s]) is/are capable of eliciting antibody response.
- **Haptens**—Small molecules that, by themselves, are not capable of eliciting an antibody response but are capable of eliciting an immune response when attached to a large carrier such as a protein. Antibodies produced to a hapten–carrier adduct also may bind to the small-molecule hapten in a specific interaction.
- **Complements**—Companion molecules that, under certain conditions, aid in the functionality of antigen–antibody complexes but are not required for antigen–antibody or hapten–antibody interaction.
- **Antibodies**—Proteins with regions that impart a high degree of specific binding to antigens (and haptens). The structural elements of an immunoglobulin G (IgG) antibody are shown in *Figure 1*.

In addition to these components, ITMs require some means to detect or monitor the binding reaction between the antigen and antibody.

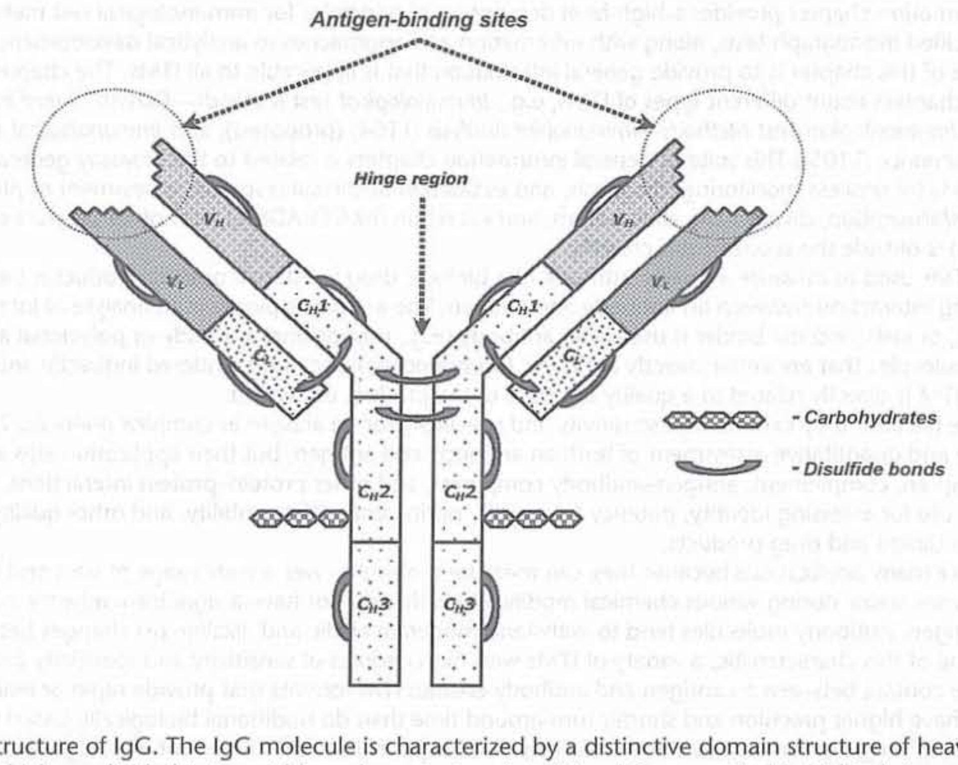


Figure 1. The structure of IgG. The IgG molecule is characterized by a distinctive domain structure of heavy (H) and light (L) chains, both of which are divided into variable and constant regions (V and C, respectively). Light chains consist of V_L and C_L domains, and heavy chains consist of a variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3). All domains are stabilized by disulfide bonds, and C_H2 domains contain carbohydrates. The flexible hinge region between the C_H1 and C_H2 domains allows the independent behavior of two antigen-binding sites formed by variable domains.

TYPES OF ITMs

Measurement of antigen–antibody binding can be performed in a variety of assay types and formats: solid or liquid phase, manual or automated, labeled or nonlabeled, competitive or noncompetitive, qualitative or quantitative, homogeneous or heterogeneous, or combinations of some of these. The distinguishing characteristic of all these assays is the binding of an antibody or antigen to the analyte (which can be an antigen or antibody as well), followed by detection of the antigen–antibody complex. Although many different formats can be used for the binding reaction, along with different methods for detection, quantification of the analyte in the test article is always performed by comparison of the measurement to a reference standard. Thus a number of ITM technologies support investigations of product quality. Commonly used assay designs include enzyme-linked immunosorbent assay (ELISA), Western blotting, flow cytometry, competitive enzyme-linked immunosorbent assay, surface plasmon resonance (SPR), rate nephelometry, radioimmunoassay (RIA), radial immunodiffusion, precipitation, and agglutination. These methods are described below.

Enzyme-Linked Immunosorbent Assay

An ELISA is a quantitative, solid-phase immunological method for the measurement of an analyte following binding to an immunosorbent and its subsequent detection using enzymatic hydrolysis of a reporter substrate either directly (analyte has enzymatic properties) or indirectly (e.g., horseradish peroxidase– or alkaline phosphatase–linked antibody subsequently bound to the immunosorbed analyte). The analyte usually is quantitated by interpolation against a standard curve of a reference material. General information chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩ discusses ELISA in greater detail, including ELISA development for quantitative analysis.

Western Blotting

A Western blot is a semiquantitative or qualitative method for measurement of a protein analyte that has been resolved by polyacrylamide gel electrophoresis and subsequently transferred to a solid membrane (e.g., nitrocellulose, nylon, or polyvinylidene difluoride). Detection can be achieved directly by reacting with a labeled primary antibody (antibody specific to the analyte of interest) or indirectly by reacting labeled secondary antibody (antibody against the primary antibody) to the primary antibody bound to the membrane-immobilized antigen. The label can be a radioisotope or an enzyme that uses the substrate to produce color, fluorescence, or luminescence. This method is semiquantitative, especially when proteins are present in low concentration and in very complex mixtures. It is commonly used in early process development (e.g., antibody screening, protein expression, protein purification, etc.). Western blotting is a powerful method for analyzing and identifying proteins in complex mixtures, particularly after separation using 2-dimensional gel electrophoresis, which separates proteins based on size and charge (pI).

Flow Cytometry

Flow cytometry is a laser-based semiquantitative technology that permits measurement of fluorophore-conjugated probes as they interact with their respective ligands on cells or particles. More details for flow cytometry can be found in *Flow Cytometry* ⟨1027⟩.

Surface Plasmon Resonance

SPR is a quantitative method for measurement of an analyte in a sample where the antibody–antigen complex formation can be measured in real time at the interface of a liquid and solid (e.g., gold surfaces or particles). The measurement taken is the real-time change in refraction of a polarized light and occurs during the formation of the antibody–antigen complex, resulting in changes to the plasmon resonance minima (i.e., the sensorgram). The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay. More details for SPR can be found in general information chapter *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩.

Rate Nephelometry

Rate nephelometry is a quantitative method for measurement of an analyte in a sample in solution by measuring the light scatter introduced by small aggregates formed by the antigen–antibody complex. The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay.

Radioimmunoassay

RIA, a sensitive ITM first developed in the 1950s, is a quantitative method for measurement of an analyte in a sample. RIA usually uses a competitive antibody–antigen binding reaction, but it also can be used in sandwich immunoassay format, including immunoprecipitation. In competitive RIAs the analyte competes for binding with a radiolabeled (e.g., using ^{125}I or ^{3}H) reference antigen that is identical to the analyte; therefore, the analyte and the antigen both compete for binding to a fixed and limiting dilution of a specific (often polyclonal) antibody. The radiolabeled antigen is present in excess. The same unlabeled antigen in the test sample competes in binding to the same site on the antibody, which is present in a fixed quantity. Binding of the unlabeled antigen to the antibody leads to the displacement of the labeled antigen, resulting in a decrease in the radioactivity of the antigen–antibody complex fraction. To separate the antigen–antibody complex from the excess unbound antigen, the complex generally is either precipitated with a secondary antibody (or protein G) immobilized on a solid matrix (e.g., glass or resin beads) or with an already immobilized primary antibody. The quantity of analyte usually is determined by interpolation against a standard curve of a reference material, where a fixed amount of antibody and radiolabeled antigen is mixed with an increasing amount of unlabeled antigen. Hence, even a small quantity of unlabeled antigen will result in a relative quantitative decrease in total bound radioactivity.

Single Radial Immunodiffusion

Single radial immunodiffusion (SRID or SRD) is a quantitative method for measurement of an analyte in a sample by measuring the diameter of the ring of precipitin formed by the antigen–antibody complex. Antigen is applied to a well in a gel infused with a constant level of antibody. Solutions with higher concentrations of antigen diffuse farther before being saturated with antibody and then precipitated. The quantity of analyte is determined by comparison to a reference standard curve measured by the same assay.

Precipitation

The underlying principle for this method is that the interaction of a multivalent antibody and antigen leads to the formation of a complex. In some cases a visible precipitate is formed. Other immunoprecipitation techniques involve the use of Protein A or Protein G beads to capture the antigen–antibody complex and facilitate the separation of the antigen–antibody complexes from the other antigens in the solution. Precipitation is not commonly used for quantitative analytical purposes because of the time required (days to complete), lack of sensitivity, and requirement for large quantities of antigen and antibodies.

Agglutination

Agglutination and inhibition of agglutination, respectively, provide qualitative and quantitative measures of certain antigens and antibodies. Inhibition of agglutination is a modification of the agglutination reaction that provides higher sensitivity to detect small quantities of proteins, chemicals, viruses, and other analytes. The principle of agglutination is similar to that for precipitation except that the interaction takes place between an antibody and a particulate antigen and leads to a visible clump or agglutination. The most common example of this application is for blood typing (i.e., A, B, or O antigen).

CHOICE OF ITM

When choosing an ITM, analysts should consider sensitivity and specificity as well as the complexity of the sample. *Table 1* provides an assay developer with a comparative view of the advantages and disadvantages of a variety of ITM formats. The intended application of the ITM should govern the choice of the most suitable format.

Table 1. ITMs Used in Biopharmaceutical Laboratories

Method	Advantages	Disadvantages	Typical Industry Uses
ELISA	• High sensitivity • Often wide dynamic range • High throughput • Low cost	• Multistage process highly dependent on proper execution of each stage • Wash steps add time and often biohazardous waste • Reagent labeling required	• Potency assessment • Specific protein concentration analysis in complex samples • Protein identification • Purity assessment • Immunogenicity assessment
Western blot	• Gives information about antigen size and/or charge • Allows separation of various antigens (or degradation/aggregation products) bearing same epitope • Can tolerate complex mixtures	• Typically works only with linear epitopes • Labor intensive • Low throughput, output • Subject to interpretation • Immobilization can alter binding • Limited to proteins	• Protein purity assessment • Protein stability assessment • Protein identity test
Flow cytometry	• High throughput • Highly automated	• Use limited to cells, particles, and samples bound to beads • Sensitive to aggregates and sample matrix	• Potency assessment • Cell identity in cell-therapy products
SPR	• Direct detection of binding • Can measure affinity precisely, including on and off rates	• Immobilization can alter binding • Regeneration can alter binding • Low throughput, output	• Immunogenicity assessment • Potency assessment • Specific protein concentration analysis in complex samples
Rate nephelometry	• Easily automated • Rapid	• Small detection range • High background for turbid samples	• Assay for individual vaccine components for check of stability and purity

Table 1. ITMs Used in Biopharmaceutical Laboratories *(Continued)*

Method	Advantages	Disadvantages	Typical Industry Uses
RIA	• Binding occurs in native conformation • Low-concentration samples can be analyzed • High sensitivity antibody used at limiting dilution that conserves reagent • Can be plate-based for higher throughput (e.g., scintillation proximity assays)	• Requires radioactive labeling for detection • Shorter half-life of some radioisotopes requires periodic preparation of the tracer • Hazardous waste	• Protein identification (e.g., hormones) • Specific protein concentration analysis in complex samples
SRD	• Precise • Simple setup	• Semiquantitative • Low precision • Low sensitivity	• Vaccine release test
Precipitation	• Low equipment cost	• Subject to interpretation • Slow • Poor sensitivity (µg range)	• Vaccine identification
Agglutination	• Rapid • Low equipment cost	• Subject to interpretation • Slow • Low specificity because of interfering substances	• Vaccine identification

KEY CONSIDERATIONS IN ITM DEVELOPMENT

The goal during method development is to produce an accurate assay that is practically feasible and possesses an acceptable degree of intra- and inter-assay precision. To minimize the overall imprecision, the sources of variability should be identified and minimized.

Reagent Selection

Immunoassays are subject to several sources of interference such as cross-reactivity, endogenous interfering substances, buffer matrices, sample components, exposed versus masked epitopes, conformation changes in the antigen of interest, and other factors. Hence, during method development, analysts must identify possible sources of interference both to develop a robust method and to aid future troubleshooting.

Cross-reactivity is a major obstacle during immunoassay development. This arises when the specificity of an antigen–antibody reaction is compromised by the cross-reactivity binding of structurally similar molecules with the reaction binder. Some common examples are protein isoforms, degraded analyte entities, molecules of the same class, precursor proteins, metabolites, etc. Cross-reactivity can be minimized by rigorous reagent characterization and selection.

Reagents used in ITM applications generally fall into one of two categories: critical reagents and noncritical reagents. Critical reagents are specific and unique to the particular ITM or reagents that are intolerant of very small changes in composition or stability. Examples of critical reagents generally include assay-specific antibodies and reference or method calibration standards. Equivalence in the assay format must be established before replacement with a new lot. Noncritical reagents are those that can vary to some degree in composition without adversely affecting ITM performance. Reagents are often assumed to be noncritical (e.g., buffers, water quality, blocking buffer, or substrate) but later may be identified as critical components if assay ruggedness fails and troubleshooting of ITM reagents begins. ITM-specific reagents, including vendor and catalog number, should be defined in test procedure documents.

Antibody selection is critical for development of a successful immunoassay because it defines the assay's specificity and sensitivity. Furthermore, during antibody generation, analysts should ensure that the immunization protocols support the end use of the antibodies. For some applications a more specific antibody can be generated by the selection of a small and specific immunogen and affinity purification of the antibody, resulting in highly defined epitope coverage. In other applications it may be critical to ensure broad coverage of the different available epitopes on the molecules of interest, and a polyclonal antibody (pAb) pool may be the best choice. Currently, monoclonal antibodies (mAb) are preferred for some applications for the detection of single analytes because of their high specificity, lot-to-lot consistency, and indefinite supply. Compared to polyclonal antibodies, mAb have a higher initial cost to produce, but for these applications, the advantages generally outweigh the initial cost. Other applications may require more comprehensive epitope selection to ensure that subtle changes in the molecule(s) do not prevent recognition of the entire antigen, and thus a pool of monoclonal antibodies, or a pAb pool, would be the preferred choice. The latter are widely used for detection in a complex mixture of analytes (e.g., host-cell proteins). Similarly, immunoassays may use two distinct epitopes on an antigen—one for capture and the other for detection—which greatly reduces cross-reactivity. Another approach to minimize cross-reactivity is to purify the antigen before immunoanalysis. Variations in incubation temperature and time can affect the reaction kinetics of antibody interactions with similar yet different antigens. Thus this property should be optimized to increase the specificity of antigen–antibody interactions.

Development of Immunoassays

Development is an important stage in the establishment of a suitable ITM. During development of an ITM, analysts explore various settings of assay parameters and interactions between parameters to identify conditions under which the assay will consistently produce reliable results using minimal reagents, effort, and time. In Quality by Design terminology, the "possible operating space" is the collection of settings of assay parameters explored, and the "design space" refers to the conditions under which the assay performs well. The necessary performance properties of the ITM (precision, accuracy, specificity, etc.) required depend on the intended use(s). During ITM development, analysts should consider the following:

- Antigen–antibody ratio;
- In sandwich immunoassays, the ratio of capture antibody to detector antibody;
- Antigen–antibody reaction kinetics in the sample matrix (antigen–antibody binding generally is not linear);
- Selection of the standard (full-length antigen for the standard or just a small portion of the antigen containing the antibody-binding epitope, among other considerations); and
- Matrix effects.

The use of design of experiments (DOE) is strongly recommended, and different DOE methods may be appropriate in each stage of development. Early in development, screening designs are particularly useful (generally two-level geometric fractional factorial designs). After screening (with a modest number of factors to study), full factorials or response surface designs are often appropriate. As development activities shift to qualification (ideally, if not typically, as the focus shifts to robustness), robust response surface designs often are a good choice. During qualification or validation, analysts may find it practical to simultaneously study robustness to assay operating conditions (using a small geometric fractional factorial) and validation parameters such as precision (via nested or crossed designs for random factors associated with repeatability, intermediate precision, and reproducibility).

Experiments that assess dilutional linearity and components of specificity, including matrix effects, usually involve construction of spiked samples. Although spiking often is performed in a dilution matrix, spiking a collection of actual samples or mixing actual samples is an important component of demonstrating robustness of dilutional linearity and components of specificity to the sample and matrix components.

Reagent Considerations

A procedure for qualifying reagent sources and vendors (including audits), ordering, receiving, and disposing of commercial reagents and consumables should be outlined in a standard operating procedure (SOP). The preparation of internal reagents must be documented in a manner that allows reconstruction. Commercial and internally prepared reagents must be labeled with identity, concentration, lot number, expiration, and storage conditions. The stability and assignment of expiration dates for internally prepared reagents often are based on available literature and scientific experience, but analysts may need to confirm these empirically. An SOP for extending expiration dating of critical reagents is recommended. In addition, analysts should implement a mechanism for reagent tracking and linking lot numbers to analytical run numbers. Unacceptable reagent performance is detected by tracking QC samples. Shifts in QC samples should prompt a review of analytical runs and changes in reagent lot numbers or review of possible deterioration of critical reagents. To avoid such shifts, analysts can cross-validate critical reagent lot changes.

The impact of collection and storage containers on analytical performance often is overlooked. When defining the stability and expiration of in-house reagents, analysts should record information about the storage container vendor, catalog, and lot number. The importance of a suitable reference standard and its characterization cannot be overemphasized for ITMs for biological products. Because of their inherent complexity, reference and calibration standards of macromolecular biologics often are less well characterized than are conventional small-molecule drug reference standards. If the calibration standard represents a mixture of different antigens (e.g., host-cell proteins), it should be shown to be representative of the antigen profile in the samples being tested. Consistency in ITM results depends on the availability of a suitable representative reference standard material.

VALIDATION

Analytical validation involves the systematic execution of a defined protocol and prespecified analysis that includes prespecified acceptance criteria. A validation demonstrates that an analytical method is suitable for one or more intended uses [see *Validation of Compendial Procedures* ⟨1225⟩, *Biological Assay Validation* ⟨1033⟩, and ICH Q2(R1)]. Qualification may involve similar or identical experiments and procedures as validation, but qualification does not require prespecified protocols, analyses, or acceptance criteria. In certain situations (e.g., use of a commercial kit), assay development may not be required before qualification. General information chapter ⟨1225⟩ discusses which assay performance characteristics must be examined during validation for four primary categories of intended uses. For example, analytical procedures that quantitate major bulk drug substances or active ingredients may not require validation of the detection and quantitation limits but do require validation of accuracy, precision, specificity, linearity, and range.

System Suitability or Assay Acceptance Criteria

The purpose of system suitability or assay acceptance criteria is to ensure that the complete system—including the instrumentation, software, reagents, and analyst—is qualified to perform the intended action for the intended purpose. All processes should be controlled by well-defined SOPs that ensure consistency, reduce errors, and promote reproducibility of laboratory processes. Training files for all personnel should be contemporaneous and should include some demonstration that analysts are qualified to perform the method and the specific ITM.

Instrument and software qualification begins with a definition of the design qualifications, including a risk assessment and gap analysis that identify potential threats to the collection, integrity, and permanent capture of ITM data. Qualification also includes installation qualifications (IQ) and operational qualifications (OQ). Purchased commercial instrument validation packages may require modification to meet the intended use at each facility. Instrumentation and software should be continuously monitored for acceptable functionality by performance qualification (PQ) and software validation test script reviews. Routine instrument maintenance is performed according to the manufacturer's recommendations, and additional maintenance may be required based on specific needs in the working environment. A complete history of routine and nonroutine instrument maintenance should be archived for each instrument. Software updates should be handled with change control and typically require additional validation. Adherence to 21 CFR 11 should be maintained.

To ensure robustness, establish a defined process for implementing new ITMs in the laboratory. Control documents should be in place, including method validation plans containing a priori method acceptance criteria and validation reports for the establishment of a new ITM. Well-written analytical test method documents are needed to ensure reconstruction of analytical results and to minimize laboratory errors.

Analytical test methods should include acceptance criteria for critical aspects of the assay, including the performance of the calibration curve, quality controls, agreement between sample replicates, procedures for repeat sample analysis, and identification and treatment of outliers, when applicable. Furthermore, an SOP should be implemented for unexpected event investigation and resolution.

DATA REPORTING

Units of Measurement

Quantitative ITMs generate test sample data with an estimated concentration based on a calibration curve fit to reference (or standard) samples using an appropriate mathematical model. When determining the amount of analyte in a manufacturing process, analysts often express the unit of measure in terms of mass of analyte per volume of solution (concentration) or mass of analyte per mass of product (e.g., parts per million). Depending on the nature of the measured analyte, the degree of measurement standardization, the geographic region, and the history of the method, analysts may express concentration in terms of weight per volume, mole per volume, or weight of analyte per weight of product. In some circumstances, concentration may be converted to an activity unit of measure in which the analyte mass is assumed to be 100% active. In certain circumstances, qualitative analysis using a predetermined cut-off value may be an acceptable alternative to quantitative methods.

Immunoassay Data Analysis

ITMs employ calibration curves prepared with reference standards of known (nominal) concentrations and are included in every bioanalytical method. This helps control variation associated with repeatability, intermediate precision, and reproducibility and permits the estimation of results for unknown test samples. Common simple statistical analyses assume that the (possibly transformed) data are normally distributed, have constant variance, are independent, and that an appropriate model has been used. For many assays, one or more of these assumptions may be inappropriate. Analysts should assess these assumptions using a substantial body of data (typically tens of assays). When these assumptions are not reasonable, the analysis becomes more complex.

Calibration curves generally are characterized by a nonlinear relationship between the mean response and the analyte concentration and typically are plotted in a log-linear manner with the (possibly transformed and/or weighted) response variable (ordinate) plotted against the nominal calibrator concentration (abscissa) in log scale. The resulting curve that encompasses the assay's validated range is inherently nonlinear and often has a sigmoid shape with horizontal asymptotes at very low and high concentrations of analyte. Competitive ITMs have a negative slope, and noncompetitive ITMs are characterized by a positive slope. The analyte concentration in a test sample is estimated by inverse regression against the calibration curve. The final result often is obtained after multiplication of the estimated concentration in the assay by a dilution factor that is required to yield a response within the ITM's quantification range.

Under the guidance of a qualified biostatistician, analysts can implement outlier tests in controlled documents that permit the exclusion of spurious sample results. A well-defined procedure should be in place regarding how to identify, repeat, and report outliers. Outlier tests and interpretation of results are described in *Analytical Data—Interpretation and Treatment* ⟨1010⟩. Test results that fall outside of their predefined specifications or acceptance criteria should be evaluated by an out-of-specification investigation to identify a root cause.

Trending

A quality system includes monitoring of ITM performance by collection and review of ITM performance characteristics. Trending may detect shifts in assay performance that may be related to events such as assay reagent lot changes, addition of new analysts, shifts in environmental conditions, and others. SOPs, study protocols, analytical test methods, and decision flow charts are recommended to strictly define the handling, use, editing, rejection, acceptability, and interpretation of calibration data and test sample results for ITMs. It is not uncommon to have several raw data reviews, including peer, QC, and quality assurance review. Analysts must be able to distinguish such analytical issues from true changes in the measured analyte caused by changes or errors in the manufacturing process that have affected the product. Two of the most important outcomes of proper trend monitoring are detecting potential problems before they occur and identifying areas for corrective and/or preventive action. General information chapters *Analytical Data—Interpretation and Treatment* 〈1010〉 and *Biological Assay Validation* 〈1033〉, as well as the statistical literature, contain guidance for various trending methods. Several ITM performance characteristics could be considered for monitoring. The most common trending value is evaluation of QC samples. Ideally, one or more QC sample is available for long-term trending in sufficient quantity and with demonstrated stability so that quality aspects can be assayed in every run and across multiple manufacturing lots. As the long-term QC sample is depleted or expires, crossover comparison and establishment of a new long-term QC sample should be completed. Systematic review of QC data across assays assists in troubleshooting failed ITM runs, providing confidence in the evaluation of spurious results, and controlling the introduction of replenished assay components that may not perform exactly like previous reagents.

Other ITM performance characteristics that may be monitored include calibration curve response variables, curve fit parameters, assay background, and comparison of in-study QC data with validation data.

Tracking

Regulatory agencies have strict requirements about maintaining the identity and integrity of both samples and data. A quality process driven by SOPs must be implemented to ensure the correct identity and integrity of test and reserve samples. Ideally, a bar code system should be used to track the collection, identity, location, chain of custody, number of sample freeze/thaw cycles, storage temperature, and length of time that a sample is stored. This information should be captured and should be auditable from the time of collection to disposal (or sample depletion). The ability to track the sample history permits reconstruction of the events leading to generation of a data result. This information is used by regulatory agencies to ensure that the proper procedures were followed and by internal auditors to ensure that pre-analytical sample handling did not compromise study data. In addition, sample tracking allows a mechanism for ensuring that the analyte measurement occurred within the demonstrated window of stability for that analyte.

The final result generated from a bioanalytical laboratory is a number that represents an analyte measurement in a test sample. The steps necessary to generate that data and preserve it in a report are numerous and are susceptible to error. Therefore, quality systems must be in place to minimize data errors. Errors may be introduced by test sample misplacement or identification, incorrect data reduction, miscalculations, transcription errors, omissions, and other factors. Ideally, validated software and laboratory information management systems are used when possible to generate, transfer, and archive data. Typically, redundancy checks are built into automated processes by visual data review of at least 10% of the data-transfer processes. In the absence of validated electronic transfer, all data should be reviewed by at least one reviewer. As with sample tracking, data generation, manipulation, and storage should be reconstructible. In addition, all data should be backed up using a format that is stable. Plans should be in place to update archived data so that, as technology changes, archived data can still be retrieved. Regulatory agencies require that raw data be available for various lengths of time after the completion of a study or regulatory filing. Finally, data must be secure from corruption, alteration, or access by unauthorized personnel.

〈1103〉 IMMUNOLOGICAL TEST METHODS—ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

INTRODUCTION

Immunological Test Methods (ITM) utilize binding between an antigen (Ag) and antibody (Ab). (See *Appendix 1* for a complete list of acronyms used in this chapter.) Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used ITM for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term ELISA is used here in a broader sense and includes enzyme immunoassays (EIA), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

This chapter provides analysts with general information about principles, procedures, experimental configurations, assay development, and validation for solid-phase ITM like ELISA and can be used for the other immunoassay variations mentioned above. The chapter also covers reference standard(s) and control(s) used for immunoassays. The information can be adapted

to the specific procedures of a monograph. This chapter does not cover immunoassays for the measurement of immune responses to product in animals or humans (e.g, serological or cellular assays), non-immunoassays (e.g, receptor-ligand interactions), or other related approaches.

The chapter is part of a group of general information chapters for immunological test methods [*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩ (proposed), and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩], and also is related to the general information chapters for bioassays [*Design and Development of Biological Assays* ⟨1032⟩, *Biological Assay Validation* ⟨1033⟩, and *Analysis of Biological Assays* ⟨1034⟩].

Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly (as with an analyte that has enzymatic properties or is directly labeled with an enzyme) or indirectly (by means of an enzyme-linked antibody that binds to the immunosorbed analyte). Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This standard should be an appropriate, preferably homologous, reference or calibration material that is representative of the analyte(s) of interest. The power of immunoassays has been demonstrated by the variety of procedures that have evolved, including alternative solid surfaces such as beads of different sorts, various plastics in plates of different configurations, and alternative detection methods, e.g., chemiluminescence and fluorescence. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

Basic Principles

The essential steps of an ELISA can be broken down as follows (see *Figure 1*):

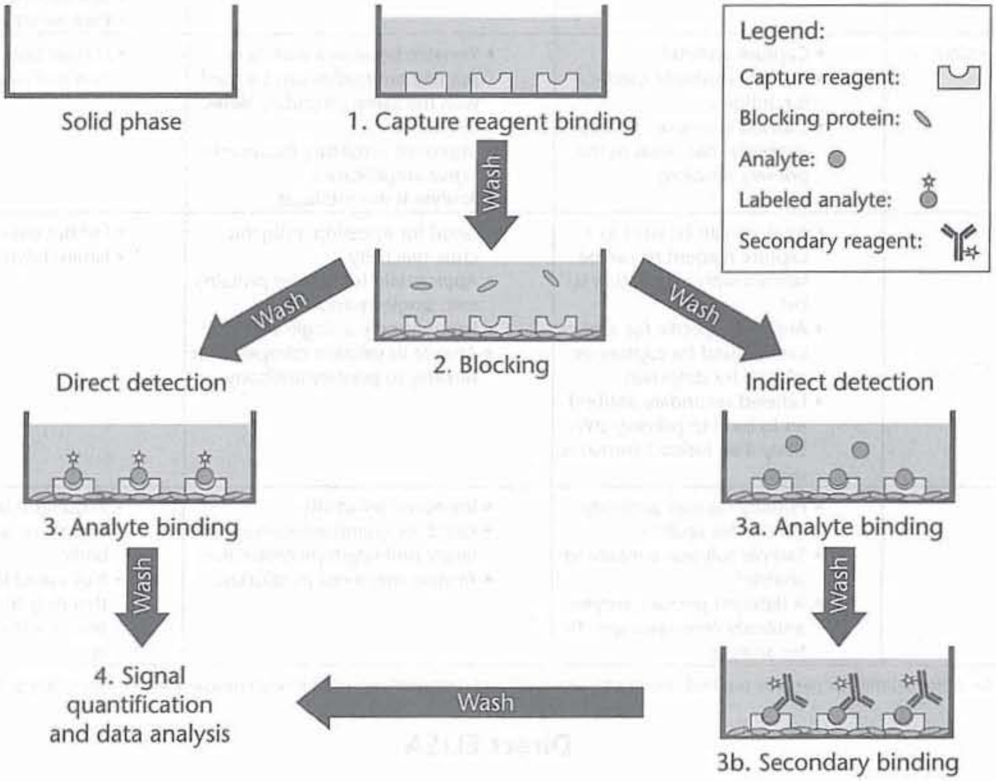


Figure 1. Essential steps for performing an ELISA.[1]

[1] Capture reagent binding, blocking, analyte binding, detector antibody binding, and analysis are the five basic steps in an ELISA. Capture reagent binding, blocking, and analyte binding steps are each followed by a washing step to remove unbound reagents before the addition of the next reagent. Before analysis an appropriate substrate is added, followed by measurement of the substrate by appropriate equipment for detection. Quantitation of unknowns takes place by comparison with a standard curve.

1. Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;
2. Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;
3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte. Direct detection occurs when the analyte has enzymatic activity or has been linked to a detector molecule (e.g., enzyme); or
3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte (*Figure 1*, step 3a). Indirect detection occurs when the analyte is detected by the addition of a secondary enzyme-labeled reagent (*Figure 1*, step 3b); and
4. Quantification of the analyte by addition of a substrate suitable for the detector used (e.g., TMB, 3,3′,5,5′-tetramethylbenzidine), followed by comparison of the test sample to the reference standard.

ASSAY DESIGN

Five general categories of ELISA are described in *Table 1* and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

Table 1. Representative ELISA Types

ELISA Type	Required Reagents	Attributes	Disadvantages
Direct detection	• Capture analyte[a] • Labeled primary antibody specific for antigen	• Rapid because only one antibody is used • Uses less reagent • Analyte is immobilized	• May modify the conformation of the analyte • Sensitive to matrix and adjuvant components • Not commonly used • Poor sensitivity
Indirect detection	• Capture analyte[a] • Primary antibody specific for antigen • Labeled secondary detector antibody that binds to the primary antibody	• Versatile because a variety of primary antibodies can be used with the same secondary detector • Improved sensitivity because of signal amplification • Analyte is immobilized	• Longer because of more incubation and washing steps
Competitive	• Analyte[a] can be used as a capture reagent or can be labeled with a detection label • Antibody specific for analyte can be used for capture or labeled for detection • Labeled secondary antibodies to bind to primary antibody if an indirect format is used	• Good for assessing antigenic cross-reactivity • Appropriate for smaller proteins with single epitopes • Requires only a single antibody • Analyte in solution competes for binding to primary antibody	• Format difficult to troubleshoot • Limited dynamic range
Sandwich	• Primary capture antibody specific for analyte • Sample solution containing analyte[a] • A different primary enzyme-antibody conjugate specific for analyte	• Improved sensitivity • Good for quantitative assays for larger multi-epitope molecules • Analyte measured in solution	• Requires relatively large amounts of pure or semipure specific antibody • Not suited for smaller proteins that may have only a single epitope or a few closely spaced epitopes

[a] This reagent can be either purified or partially purified. The terms "analyte" and "antigen" are used interchangeably when describing ELISAs.

Direct ELISA

DIRECTLY LABELED ANTIBODY

In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked (*Figure 2A*). Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away, followed by the addition of an appropriate substrate for the detector used.

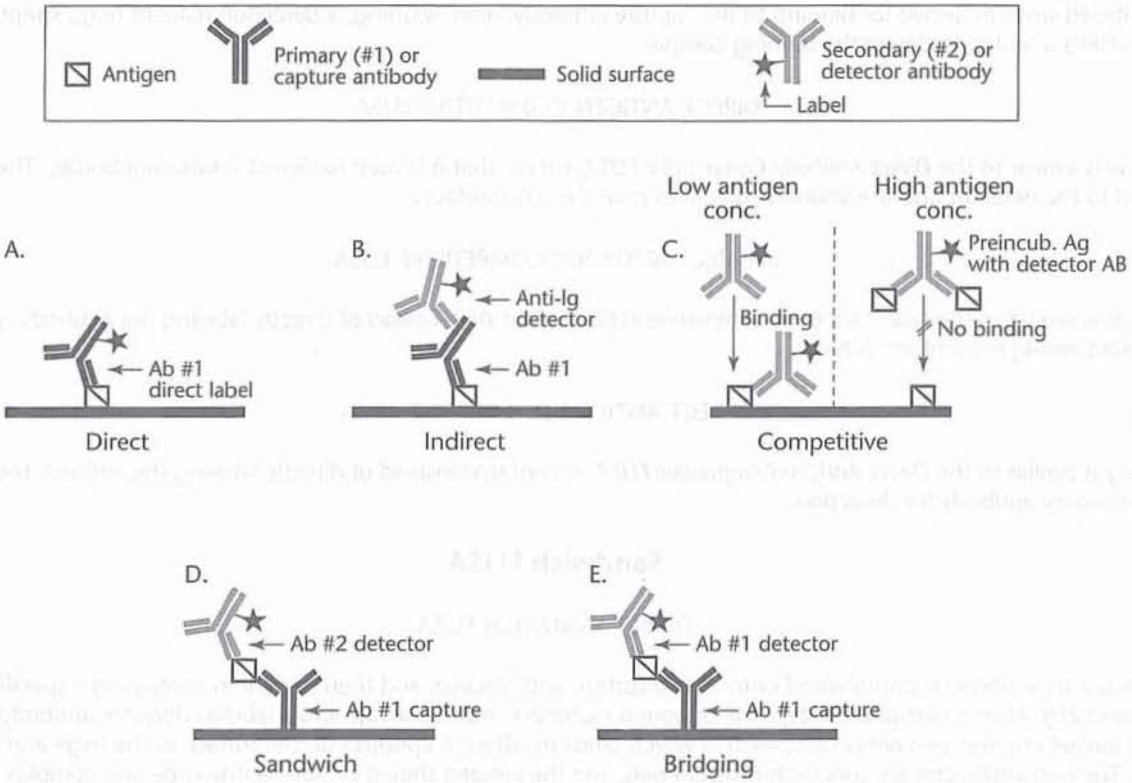


Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.[2] (Ab = antibody; Ag = antigen (or analyte); Preincub = preincubation)

DIRECTLY LABELED ANTIGEN

This assay is similar to that using a directly labeled antibody except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added (*Figure 2B*). After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a *Direct ELISA*, thereby increasing assay sensitivity.

Competitive ELISAs

DIRECT ANTIBODY COMPETITIVE ELISA

This assay is used to detect or quantitate soluble antigens (*Figure 2C*). It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase, alkaline phosphatase, ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen-antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., colorimetric, electrochemiluminescence, fluorescence, or chemiluminescence) is measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes

[2] The type of ELISA format depends on the availability of reagents, the intended purpose of the assay, and the physicochemical characteristics of the analyte of interest. For a *Bridging ELISA*, the capture and detector antibodies recognize the same epitope, and therefore the target antigen must have at least two epitopes available for binding.

with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

DIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

INDIRECT ANTIBODY COMPETITIVE ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

INDIRECT ANTIGEN COMPETITIVE ELISA

This assay is similar to the *Direct Antigen Competitive ELISA*, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

Sandwich ELISA

DIRECT SANDWICH ELISA

In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added (*Figure 2D*). After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

INDIRECT SANDWICH ELISA

Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detector could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

BRIDGING ELISA

This subset of *Sandwich ELISA* assays often uses a single antibody for both capture and detection (*Figure 2E*). If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g, an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-

channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the EC_{50} and require a wider dose range.

PROCEDURES

Solid Phase

Solid phases are available in a variety of forms (e.g, membrane, plate, or bead) and chemistries (e.g, nylon, nitrocellulose, polyvinylidine fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface). The selection of the solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

COATING THE SOLID PHASE—IMMOBILIZATION OF CAPTURE REAGENT

Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye's color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 µg/well are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 µL. Coating duration, temperature, and buffers are discussed separately below. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

MICROTITER PLATES

The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require optimization of buffer pH based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

COATING TIME

Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

COATING TEMPERATURE

Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen. Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

BUFFERS

Buffers used for diluents, coating, blocking, and washing plates can affect overall assay performance. Buffer components can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

Diluent—Buffers [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer.

Coating Buffers—Coating buffers should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffers include 50 mM carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer depends on the nature of the individual antigens and should be determined empirically.

Blocking Agents and Buffers—A blocking agent is a compound (e.g., protein or detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the dilution buffer. The choice of blocking agent should be governed by the nature of the capture reagent, plate, coating buffer, test sample diluent, and related factors. If any of these parameters changes, a change in blocking agent may be needed. Commonly used blocking agents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITMs.

Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

PIPETS

Single, multichannel, and robotic pipets with set or fixed volumes are available. The type and accuracy of pipets should be evaluated for each application. Regular maintenance and professional calibration of pipets should be performed and documented.

PIPET TIPS

A variety of pipet tips are available, some of which are specific to the type of pipet. The type and accuracy of the pipet tip, particularly related to the viscosity and nonspecific binding of the materials, should be evaluated for each application.

Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and detection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in wash buffer, and adding wash buffer with a multichannel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multiwell washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer left in the well. Incorrectly programmed or maintained as well as incompletely cleaned automatic washers can cause assay variation and elevated assay background.

Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

Blocking Conditions and Nonspecific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1. *Ionic or hydrophobic interactions* occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.
2. *Immunological interactions* occur when binding is mediated by unintended antigen-antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

The choice of blocking agent (examples are found in the *Blocking Agents and Buffers* section above) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffers that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer in place of sample. Control wells also can be useful as part of system suitability testing.

Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article's properties and/or introduce further variability that results in biased measurements. Samples, standards, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

Detector Antibodies

Depending on ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA (*Figure 2A* and *Figure 2C*), after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats (*Figure 2B, 2D,* and *2E*), the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the conjugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate controls.

The most commonly used labeling enzymes for conjugating to antibodies include alkaline phosphatase (AP), horseradish peroxidase (HRP), and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. *para*-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, OPD (*o*-phenylenediamine dihydrochloride), and ABTS [2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt] (see *Table 2*). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see *Table 2*). Well-known fluorescent substrates for galactosidase include MG (4-methylumbelliferyl galactoside) and NG

(nitrophenyl galactoside). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

Table 2 also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay's purpose and the requirements of the assay.

Table 2. Enzyme Conjugates and Substrates

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Reader	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP, HRP	pNP TMB OPD ABTS	Spectrophotometer	• Robust • Economical • Reagent availability	• Less sensitive
Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP	CSP D	Luminometer	• Wide assay dynamic range • Lower coating concentrations • More sensitive • Rapid signal generation	• Requires special plates • Costly
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase	MG NG	Fluorometer	• Rapid • Sensitive	• Requires special plates • Costly • Interference by excipients

ASSAY DEVELOPMENT AND VALIDATION PLAN

Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated (see, for example, guidance contained in *Design and Development of Biological Assays* 〈1032〉.)

SOURCE

The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

PURITY

In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

SPECIFICITY

The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific binding or no cross-binding to off-target molecules in complex test materials.

STABILITY

The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

- Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
- Understand the performance goals for the assay system.
- Develop the assay to the point that there is a detectable concentration response curve.
- Perform method development/robustness testing.
- Prepare the reference/calibration standard and control and assess stability.
- Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
- Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
- Validate the assay.
- Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
- Monitor assay performance.

During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs.

Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

Assay Validation

Assay validation is executed according to guidances from appropriate regulatory bodies (e.g., ICH Q2) to demonstrate that the particular test used for an analyte is appropriate for its intended use. More information about assay validation can be found in the general information chapter *Validation of Compendial Procedures* ⟨1225⟩ or in general information chapter *Biological Assay Validation* ⟨1033⟩ if the ELISA is used as a surrogate potency assay. See *Appendix 2* for additional information.

DATA ANALYSIS

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a nonlinear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration (IC_{50}) or effective concentration (EC_{50}), estimation of the amount of a sample that yields the same response as the EC_{50} (or IC_{50}) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard. More extensive guidance about statistical methods for potency analysis are given in general information chapters *Design and Development of Biological Assays* ⟨1032⟩ and *Analysis of Biological Assays* ⟨1034⟩.

In general, ELISA assay curves are characterized by a nonlinear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

Basic Statistical Analysis

Basic statistical methods are not detailed here. General information chapter *Analytical Data—Interpretation and Treatment* ⟨1010⟩ addresses important fundamentals, including data handling; computation of means, standard deviations, and standard errors; detection of and methods to address nonconstant or nonnormal variation; detection of and management of outliers; and procedures for and interpretation of statistical tests and confidence intervals. The concepts behind validation, goals, designs, analysis, and practical methods for validation are described in general information chapters *Analytical Data—Interpreta-*

tion and Treatment ⟨1010⟩, *Validation of Compendial Procedures* ⟨1225⟩, and *Biological Assay Validation* ⟨1033⟩. General test chapter *Design and Analysis of Biological Assays* ⟨111⟩ contains guidance on combining results from independent assays.

Nonlinear Statistical Analysis

Nonlinear calibration for immunoassays draws on many sources for statistical design and analysis. These include methods for assessing and addressing nonconstant variance, designs and analysis methods for experiments with complex structures, and validation. The concepts behind linear calibration design, analysis, and inverse regression apply in nonlinear calibration, and professional statisticians can help apply these appropriately.

Reporting Results

Reported estimates of concentration should be understood as having an associated confidence interval based on the results of the validation. The reported value or estimate used to describe a sample can be based on a combined result from multiple assays.

APPENDICES

Appendix 1: Abbreviations

Ab—Antibody
Ag—Antigen
ABTS—2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
Anti-Ig—anti-immunoglobulin
AP—Alkaline phosphatase
BSA—Bovine serum albumin
CSPD—Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate
EIA—Enzyme immunoassay
ELISA—Enzyme-linked immunosorbent assay
HRP—Horseradish peroxidase
Ig—Immunoglobulin
ITM—Immunological test methods
MG—4-Methylumbelliferyl galactoside
NG—Nitrophenyl galactoside
OPD—*o*-Phenylenediamine dihydrochloride
PBS—Phosphate-buffered saline
PVDF—Polyvinylidene fluoride
pNPP—*para*-Nitrophenyl phosphate
TMB—3,3′,5,5′-Tetramethylbenzidine

Appendix 2: Additional Sources of Information about Specific Topics in Validation and Data Analysis

	Analytical Data—Interpretation and Treatment ⟨1010⟩	**Design and Analysis of Biological Assays** ⟨111⟩	**Validation of Compendial Procedures** ⟨1225⟩	**Biological Assay Chapters** ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩
Means	X			
Standard deviations	X			
Standard errors	X			
Non-normality	X			X
Nonconstant variance	X			X
Outliers	X			X
Tests	X			
Confidence intervals	X			X
Validation			X	X

	Analytical Data—Interpretation and Treatment ⟨1010⟩	Design and Analysis of Biological Assays ⟨111⟩	Validation of Compendial Procedures ⟨1225⟩	Biological Assay Chapters ⟨1032⟩, ⟨1033⟩, and ⟨1034⟩
Combining results from multiple assays		X		X

⟨1104⟩ IMMUNOLOGICAL TEST METHODS—IMMUNOBLOT ANALYSIS

INTRODUCTION

Definition and Scope

The chapter is part of a group of general information chapters for immunological test methods (*Immunological Test Methods—General Considerations* ⟨1102⟩, *Immunological Test Methods—Enyzme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩) that provides analysts with general information about principles, method development, method validation, and data evaluation for immunoblot analysis. Immunoblot analysis is defined as any method in which an antibody is used for detection of one or more analytes (e.g., proteins, polysaccharides) that has been transferred to a test membrane surface. Immunoblot methods are typically classified by whether electrophoretic separation occurs as a part of the immunoblot procedure. Electrophoretic separation is based on molecular weights and charge differences of a population of molecules. See *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩ for a detailed description of electrophoretic separation methods. An example of immunoblot analysis involving electrophoretic separation is the Western blot, which was first described in the scientific literature in the late 1970s. Another approach for immunoblot analysis is to perform molecule detection using an antibody without prior electrophoretic separation. Examples of this nonelectrophoretic type of approach are the slot or dot blot (slot/dot).

The scope of this chapter includes only those methods in which an antibody is used for the detection of a molecule bound to a membrane. Therefore this chapter does not discuss procedures that use nonimmunological means of detection.

ASSAY SELECTION

Nonelectrophoretic Assay (Slot/Dot Blot)

The slot/dot blot method is a simplified, nonelectrophoretic method in which a mixture containing the analyte(s) for detection is first applied directly to the membrane using a vacuum manifold machined to contain regularly spaced rectangular slots. The slot/dot blot method is faster and simpler because there is no electrophoretic separation of the multiple, individual analytes that may be present in the mixture. For these reasons, it can be readily adapted for automated analysis of multiple samples, for which a number of systems are commercially available, but it offers no information about the molecular weight and only limited information regarding the quantity of sample. Although it can be set up in a quantitative format, the method usually is used to produce a qualitative result, e.g., confirming identity by demonstrating the presence or absence of specific antigens by means of an immunocomplex detection system. After the analytes are bound to the membrane and unbound sites are blocked, analysts use a detector antibody to determine the presence or absence of the analyte or analytes of interest. The uniform shape of the slot blot and its greater surface area for analyte binding make it better suited than the dot blot for quantitative applications and analysis by densitometry.

Electrophoretic Assay

Electrophoretic blotting methods (commonly called Western blots) are widely used for analyzing mixtures of proteins. The Western blot is a powerful tool to study the identification, relative concentration, relative molecular weight, and posttranslational modifications of specific proteins. In Western blots, the proteins of the sample are separated using gel electrophoresis. Protein separation may be based on molecular weight alone or on isoelectric point (pI) and molecular weight. Proteins migrate either in one dimension (1D) or in two dimensions (2D) through a gel. When proteins are separated by their molecular weights, the smaller proteins migrate faster and separate according to molecular weight. When analysts use a 2D gel, proteins are separated according to pI in the first dimension, and then according to their molecular weights in the second dimension. After separation, the proteins are transferred to a membrane, the membrane is blocked to avoid nonspecific binding of subsequent assay reagents, and the protein of interest is detected using specific antibodies.

A bound antibody can be detected by different methods, including colorimetric detection, fluorescent detection, chemiluminescent detection, and radioactive detection. Upon detection of the protein(s) of interest, immunoblot quantitation can be indirectly performed by densitometry.

1D ELECTROPHORESIS

In 1D electrophoresis, individual proteins or groups of proteins are separated by molecular weight for further analysis by Western blot. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins migrate in response to differences in electrical charge through a 3D network of fibers and pores. The network is formed as the bifunctional bisacrylamide, or other cross-linker, cross-links adjacent polyacrylamide chains to form a gel (see also *USP* general chapter ⟨1056⟩). The combination of gel pore size and protein characteristics determines the migration of proteins. Separated proteins are detected subsequently by Western blot analysis using antibodies specific to the target proteins. By means of Western blot analysis, a test sample can be compared to a standard, and the appearance of degradants and impurities specifically related to the target proteins can be monitored if the detection antibody can still recognize the altered forms of the protein. Although a high level of sensitivity can be achieved by this approach, separation of individual proteins at similar molecular weights may not be achieved. If analysts must probe individual proteins at similar molecular weights, 2D separations may be required.

2D ELECTROPHORESIS

In 2D electrophoresis, individual proteins or groups of proteins are separated in the first dimension by isoelectric focusing (IEF; charge) and in the second dimension by electrophoresis in the presence of SDS (molecular weight). Separating proteins this way allows information to be obtained not only about molecular weight, as in 1D gels, but also about the charge of a protein. Two-dimensional gels are a useful choice for resolving complex mixtures and for assessing protein antibody specificity (e.g., evaluation of host cell proteins).

Membrane, Reagent, and Detection Options

MEMBRANES

Generally, both nitrocellulose and polyvinylidine fluoride (PVDF) membranes are used for immunoblot methods. For cost considerations, nitrocellulose membranes are often preferred over PVDF membranes for slot/dot blots (or vacuum blotting), but due to their greater mechanical strength, PVDF membranes should be considered if stripping and reprobing are required.

BLOCKING REAGENTS

Following transfer or binding of protein to membranes, the unoccupied binding sites on the membranes must be blocked to prevent nonspecific binding of subsequent reagents. Most detection probes are proteins that also can bind to the membrane. Failure to appropriately block the membrane sites can result in nonspecific binding and high background. A number of blocking reagents are available, including gelatin, nonfat milk, and bovine serum albumin (BSA). Proteins should be unrelated to the antigens used in the study. Because these reagents often have lot-to-lot variability, they may require qualification. They must be evaluated with the detection system selected and optimized using that detection system for minimal background with no loss of signal. If the blocking reagent is derived from a biological source, it must not contain trace levels of the protein under measurement, because the latter can increase the background.

METHODS OF DETECTION

Immunological detection of analytes in any type of immunoblot can be direct or indirect. The choice of format depends on a combination of the level of sensitivity required and the quality of the antisera available. For identity or product detection, sensitivity usually is not critical, and direct detection via a conjugated antibody is commonly used, which often simplifies and shortens the time required to execute the method. Alternatively, indirect detection, usually by the use of a conjugated anti-species reagent, can be used to improve sensitivity. On some occasions, the analyte being detected is actually an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, antibodies specific for the antibody (e.g., anti-idiotypic antibodies) can be used.

Primary antibody: The primary antibody is selected based on its specificity for the analyte or protein. Although polyclonal anti-sera can offer a broad range of detection against a potentially large set of epitopes, an unwanted cross-reaction resulting in decreased specificity may occur. If this cannot be overcome by assay optimization, a monoclonal antibody or groups of selected monoclonal antibodies can be used. Monoclonal antibodies are often advantageous for long-term studies, because they yield a consistent supply of antibody against a specific epitope. The use of monoclonal antibodies directly limits the number of epitopes involved in the detection of the target. This must be evaluated for each application. The primary antibody may be directly conjugated or used in conjunction with a secondary antibody and an appropriate detection system. The optimal anti-

body concentration usually is considered to be the greatest dilution of antibody that results in a strong positive signal with minimal background. This must be optimized in conjunction with the block and detection system selected. The primary antibody should be qualified before assay use.

Secondary antibody: The secondary antibody typically is directed against the species of the primary antibody immunoglobulin (which is specific for the analyte, e.g., goat anti-mouse IgG). Enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) typically are linked to the secondary antibody, but other labels such as fluorophores or gold particles can be used for detection. If the secondary antibody is biotinylated, biotin–avidin–HRP or –AP complexes can be used for detection.

Detection enzyme and substrate: Once an immunocomplex containing the enzyme–conjugate reagent has formed, analysts add a suitable substrate to the assay. This reaction results in production of a colored precipitate or a fluorescent or chemiluminescent product that can be recorded, measured, and analyzed further. A broad range of detection options is available to best fit individual applications and intended uses. A number of these are described in ⟨1103⟩ and ⟨1102⟩, as well as in *Table 1*.

Table 1. Detection Reagents and Methods

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Detection	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP[a] HRP[c]	pNPP[b] TMB[d] OPD[e] ABTS[f]	Spectrophotometer	— Robust — Economical — Reagent availability	— Time-consuming — Less sensitive than other methods
Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP, HRP	CSPD[g]	Luminometer, photographic film (CCD[h] camera)	— Wide assay dynamic range — Very sensitive — Rapid signal generation	— Reproducibility can be challenging
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase, fluorescently labeled antibody	MG[i] NG[j]	Fluorometer (CCD camera with filters)	— Rapid — Sensitive	— Interference by excipients
Radioactive	Antigen is labeled with a radioactive isotope. Radiation is proportional to analyte concentration.	—	—	Scintillation counter	— Easy to quantitate — Rapid	— Safety risk with exposure — Radioactive waste

[a] Alkaline phosphatase.
[b] *para*-Nitrophenyl phosphate.
[c] Horseradish peroxidase.
[d] 3,3′,5,5′-Tetramethylbenzidine.
[e] o-Phenylenediamine dihydrochloride.
[f] 2,2′-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt.
[g] Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate.
[h] Charge-coupled device.
[i] 4-Methylumbelliferyl galactoside.
[j] Nitrophenyl galactoside.

METHOD DEVELOPMENT

Method development can proceed on the basis of the background information just provided. The scope of method development, and eventually method validation, are dictated by the purpose of the method. The purpose determines the format for the assay and other requirements for the test, and therefore, the purpose should be determined first. The following sections explore considerations for each method's purpose.

Intended Purpose of the Method

IDENTITY TESTING

In the case of identity tests, analysts want to detect the presence of a protein; therefore, demonstration of specificity is essential and required. For this purpose, analysts also control the quantity of protein in the sample. Thus, the limits of detection (LOD), limits of quantitation (LOQ), and other measures of quantity are not required attributes of the method. Examples include material identity assays that demonstrate the isotype of an IgG and, in some cases, demonstrate the specificity of an antibody in a method validation. If there is no interference from the matrix or potential cross-reaction with other materials present in the sample, then a simple slot/dot blot may suffice. If multiple proteins in the sample display immunoreactivity and must be distinguished from each other, another separation procedure must be used before blotting and immunostaining. The

complexity of the proteins in the sample and the usefulness of the additional information gained using an electrophoretic separation help determine if a slot/dot blot can meet the needs of the test.

LIMIT TESTING

In other applications, analysts may want to show that an impurity has been removed to a level below toxicological concern. In many cases, a limit test is used when it is possible to say *yes* or *no* about the presence or absence of a protein below a predetermined level. This simplifies the development and validation of the method. With densitometry (scanning or imaging) equipment, the intensity of spots or bands can be determined relative to a standard curve, resulting in an estimate of concentration. An LOD should be determined to establish the appropriate limit threshold for the method. A dot blot may be suitable for either circumstance if the specificity of the antibody in the sample matrix can be demonstrated.

Another common purpose for an immunoblot is to show the presence or absence of a protein expressed from a culture. In this situation, analysts want to establish the identity of the protein by immunostaining, as well as verify that the protein has the expected molecular weight. This provides further assurance of no nonspecific interactions with other proteins in a complex matrix that generates the signal in the blot.

SPECIFICITY TESTING

Characterizing the specificity of the reagents for an ELISA impurity test or an immunoaffinity column also is a common immunoblot purpose. This is another form of an identity test in which the desired endpoint is demonstration of the specificity of binding between the antigen and the antibody. The result of the measurement is a demonstration of binding to a select group of the total protein population in the sample or binding to the whole population of proteins in the sample, as required for host-cell protein assays. In order to demonstrate the specificity of an antibody relative to a population of proteins, analysts typically must carry out electrophoretic or other separations. Showing, by means of immunostaining, that a protein of the right molecular weight or pI can be recognized by the antibody is a powerful demonstration both of specificity toward a given protein and the absence of binding to other proteins. In addition, having, within the same experiment, the appropriate positive control samples that are known to contain the protein, and the appropriate negative samples that are known not to contain the protein, makes a convincing argument for the specificity and selectivity of the antibody when analysts validate an ELISA method for protein impurities. Electrophoretic separations can be done in one dimension using either SDS–PAGE (for molecular weight) or isoelectric focusing (IEF; for isoelectric point) for a limited number of proteins with known molecular weights. Electrophoretic separations also can be done in two dimensions (e.g., IEF followed by SDS–PAGE) to show selectivity and specificity toward a more heterogeneous population of proteins. A 2D Western blot commonly is used to demonstrate the specificity of a polyclonal antibody candidate directed against a host-cell protein (HCP) antigen preparation before development of a quantitative ELISA for that purpose.

Assay Mode and Sample Introduction

After considering the critical elements required for each purpose of the method, the analyst can use this information to select the most appropriate assay mode. The main points to consider when developing an immunoblot method are shown in *Figure 1*. If appropriate, spotting samples on a membrane or applying by vacuum is the easiest and most convenient way to introduce a sample to an immunoblot membrane. However, low levels of nonspecific binding from multiple proteins can create additive nonspecific interference in dot blots or slot blots, resulting in background levels that appear to be the desired analyte.

General Chapters

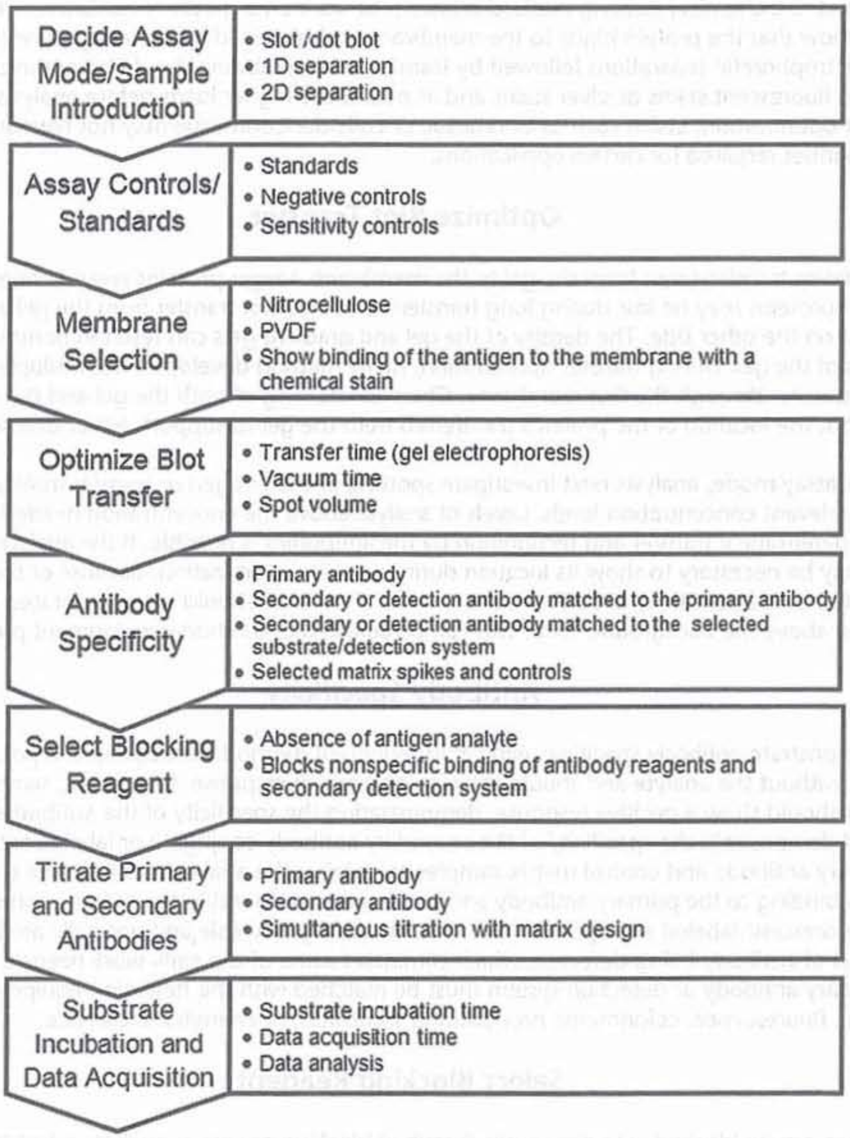


Figure 1. Method Development Flow Chart.

Electrophoretic separations, although time consuming, can be useful for separating and further distinguishing specific and nonspecific binding. Analysts must trade sensitivity for selectivity in going from a single dimension to two dimensions because of the further separation of immunoreactive species from a single band into multiple spots, as is the case with the heterogeneity seen in sialylated proteins or deamidated species.

Assay Controls and Standards

Controls and standards are selected based on the purpose of the assay and the information needed during development. Protein molecular weight markers can be used to obtain an accurate estimate of the molecular weight of immunoreactive species. The use of positive and negative controls is helpful for troubleshooting throughout the experimental design process. Standards or positive and negative controls can be used to assess system suitability and to establish method performance. A positive control can confirm appropriate protein migration and can confirm that membrane transfer has reached completion. A negative control is useful for assessing nonspecific interactions. A method sensitivity control near the LOQ can be used to measure the consistency of the method near the LOQ to evaluate changes in assay performance.

Membrane Selection

A membrane is selected based on the application and the protein being measured. Membranes with various pore sizes should be available and should be suitable for the molecular weight of the protein of interest to aid in appropriate transfer of

different sizes of proteins. If a chemical staining method is known to work on a specific membrane with a specific protein, then it is advantageous to show that the protein binds to the membrane and is stained before analysts work on the immunostaining steps for the assay. Electrophoretic separations followed by transfer to a membrane should be optimized with chemical staining, e.g., with sensitive fluorescent stains or silver stains and at potentially higher loads before analysts work on lower load levels required for blot optimization. Stains such as Coomassie or colloidal Coomassie may not have sufficient sensitivity to detect a low level of impurities required for certain applications.

Optimize Blot Transfer

Analysts should optimize transfer times from the gel to the membrane. Larger proteins require more time for transfer than smaller proteins. Small proteins may be lost during long transfer times and can transfer from the gel all the way through the membrane and be lost on the other side. The density of the gel and gradient gels can result in nonuniformity of transfer from the top to the bottom of the gel. During transfer optimization, many method developers use multiple membranes in order to capture proteins that transfer through the first membrane. Chemical staining of both the gel and the membranes can provide useful information about the location of the proteins transferred from the gel to support, either extending or reducing the transfer time.

After they select the assay mode, analysts next investigate spotting of the antigen or transfer from a gel to the appropriate membrane at various relevant concentration levels. Levels of analyte above the concentration needed for a Western blot may be required at first to determine if transfer and recognition by the antibodies is possible. If the analyte is present in low concentrations, spiking may be necessary to show its location during transfer optimization. Because of the potential variability of immunostaining and transfer, a sensitivity control or several levels of controls should be incorporated into the method based on the analyte titration above the background level. This can be adjusted as method development progresses.

Antibody Specificity

Analysts should demonstrate antibody specificity early in immunoblot method development. If possible, they should test samples of the matrix without the analyte and should show an absence of response. In contrast, samples that contain analyte spiked into the matrix should show a positive response, demonstrating the specificity of the antibodies.

Analysts also should demonstrate the specificity of the secondary antibody conjugate or label. Control immunoblots with lanes or spots of primary antibody and control matrix samples containing the analyte as a negative control can show that the secondary antibody is binding to the primary antibody and not to proteins found in the matrix. Commercial sources for enzyme conjugates or fluorescent-labeled anti-species antibodies are readily available and normally are screened or affinity purified against the species of antibody being detected, which eliminates some of the early work needed to achieve the desired specificity. The secondary antibody or detection system must be matched with the detection equipment and the desired sensitivity of the assay, e.g., fluorescence, colorimetric precipitating substrates, or chemiluminescence.

Select Blocking Reagent

Replicate membranes can be blocked with previously described blocking agents as analysts select the most appropriate blocking reagent and the amount of time required to minimize background by means of subsequent primary and secondary antibody incubations. Analytes titrated at multiple concentrations on the membrane allow analysts to assess the amount of signal to the amount of noise (background) with various blocking reagents followed by immunostaining with the primary antibody, labeled secondary antibody, and substrate, if needed, for visualization. This titration also serves as the starting point for examining LOD and LOQ for limit tests and quantitative measurements. The LOD for immunoblots is determined by the level of nonspecific background relative to the specific signal from the analyte. As is the case with any other analytical method, if the background and signal are equal, there is no distinction between the signal and the noise.

Titrate Primary and Secondary Antibodies

Titrating the level of primary and secondary antibody from low to high dilutions can also, as with a blocking reagent, be used to select an antibody concentration that reduces background binding in the blank regions surrounding protein spots or bands, and can optimize the signal from the analyte. A matrix grid that varies the level of primary signal against secondary signal can be useful for optimizing the background, improving analyte signal, and reducing the consumption requirements for expensive antibody reagents.

Immunoaffinity chromatography against a highly purified antigen can be used to reduce the level of nonspecific interference for all of the immunological reagents used in an immunoblot. The method developer must be cautious that the selectivity, specificity, and affinity of the primary antibody are not lost in affinity purification because of high-affinity antibodies that remain on the antigen column or because of the destruction of antibody binding caused by elution conditions. For the secondary antibody, immunoaffinity-purified anti-species antibodies are available commercially with a variety of possible labels conjugated to the antibody.

Substrate Incubation and Data Acquisition

Analysts can optimize substrate development time for enzymes in order to minimize background and improve the LOD and LOQ. Excessive substrate development times for precipitating substrates can result in an intensification of the background level relative to the specific signal from the desired analyte. If the blot is agitated during substrate incubation, undesired swirling patterns of product from precipitating substrates can form. Too short an incubation time results in a less-specific signal, but too long a time can result in high background and poor resolution. Most enzyme conjugates have an optimum development time. Fluorescent labels and chemiluminescent labels have the advantage of acquisition by scanning instrumentation that can store data electronically and perhaps acquire image signals in an additive manner. Fluorescent labels have the added advantages that the signal is stable with time, numerous experiments for development time can be obtained with a single blot, and optimization of signal acquisition can be performed on a single blot.

PROCEDURES

Slot/Dot Blots

Using an appropriate slot/dot apparatus, analysts can make antigens of interest adhere to a suitable membrane (e.g., nitrocellulose) by gravity or vacuum filtration, followed by addition and incubation of antigen-specific antibodies that bind to epitopes on the antigens. Remaining binding sites on the membrane are blocked by the addition of nonspecific antigen (e.g., BSA), followed by probing of the antigen-specific antibodies with a detection system [e.g., protein A/G conjugated to HRP binds to the antibodies that then are visualized using a 4-chloro-naphthol (4-CN) peroxidase substrate]. Positive identification is the development of dots or bands on the membrane. A negative result remains white or exhibits faint bands that are considerably lighter than positive bands.

1D Immunoblotting

PREPARATION OF SDS–PAGE GELS

Analysts should choose an SDS–PAGE gel with a content of acrylamide–bisacrylamide suitable for the molecular weight(s) of the protein(s) of interest; i.e., the smaller the molecular weight of the protein, the higher the percentage of mono- or bisacrylamide, and conversely, the larger the molecular weight of the protein, the lower the percentage of mono- or bisacrylamide.

Uniform-concentration gels have separation ranges as shown in *Table 2*, and gradient gels have a separation range as shown in *Table 3*. Gels can be purchased ready-made or can be produced in the laboratory according to procedures in 〈1056〉.

Table 2. Linear Range of Separation (kD) for Uniform-Concentration Gels

Acrylamide Concentration (%)	Linear Range of Separation (kD)
5	57–212
7.5	36–94
10	20–80
12	12–60
15	10–43

Table 3. Linear Range of Separation (kD) for Gradient Gels

Acrylamide (%)	Protein Range (kD)
5–15	20–250
5–20	10–200
10–20	10–150
8–20	8–150

SAMPLES AND STANDARD

To prepare samples, analysts typically must lyse cells and tissues in order to release the proteins of interest. The main consideration when choosing a lysis buffer is whether the antibody chosen for detection of the protein(s) of interest can recognize denatured samples. When this is not the case, analysts use buffers without detergent or with relatively mild, nonionic detergent.

Samples should be treated (e.g., reduced, nonreduced, or denatured) according to general chapter 〈1056〉, and when a sample of unknown protein content is used, a series of dilutions should be loaded onto the gel. Standards (molecular weight markers) should be treated according to the manufacturer's instructions.

ELECTROPHORESIS

Before applying samples to the stacking gel wells according to 〈1056〉, analysts denature samples (e.g., heat at 95°–100° for 5 min). An appropriate volume of sample is loaded onto the gel, and a voltage of 8 V/cm applied until the dye has moved into the resolving gel. Afterward, the voltage is increased to 15 V/cm, and the separation is run until the bromophenol blue reaches the bottom. If a commercially available gel is used, the manufacturer's recommendations are followed. *Table 4* shows common sample-loading volumes for particular gels.

Table 4. Common Sample-Loading Volumes

Wells	Gel Thickness (mm)	Maximum Sample Load Volume (µL)
10	1.0	25
10	1.5	37
12	1.0	20
15	1.0	15
15	1.5	25

TRANSFER

After electrophoresis, the proteins of interest can be blotted to a membrane such as nitrocellulose or PVDF with a pore size that is appropriate for the molecular weight of the proteins of interest. Both nitrocellulose and PVDF have a protein-binding capacity of about 100–200 µg/cm². PVDF is more chemically resistant than nitrocellulose and is easier to handle. Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus and vary depending on the system.

Transfer can be done in wet or semi-dry conditions. Semi-dry transfer generally is faster, but wet transfer is especially recommended for large proteins >100 kD. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

A standard buffer for transfer is the same as the buffer used for the migration or running buffer without SDS, but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kD, SDS should be included at a final concentration of 0.1%. Lowering methanol in the transfer buffer also promotes swelling of the gel, allowing large proteins to transfer more easily. *Table 5* contains common buffers used for Western blot methods.

Table 5. Common Western Blot Buffer Formulations

Buffer	Content
Sample buffer 2× (nonreducing) 1D electrophoresis	1.89 g of Tris 5.0 g of SDS 50 mg of bromophenol blue 25.0 mL of glycerol 100 mL of water Adjust with HCl to a pH of 6.8. Add water to 125 mL.
Sample buffer 2× (reducing) 1D electrophoresis	To nonreducing sample buffer: Add 12.5 mL of 2-mercaptoethanol before adjusting the pH. Alternatively, use 1.93 g of Tris, and add a suitable quantity of DTT[a] to obtain a final concentration of 100 mM DTT.
Running buffer 10× 1D electrophoresis	151.4 g of Tris 721.0 g of glycine 50.0 g of SDS Add water to 5000 mL. Adjust to a pH of 8.1–8.8.
Transfer buffer 10×	151.4 g of Tris 721.0 g of glycine Add water to 5000 mL. Adjust to a pH of 8.1–8.8.
Transfer buffer 1×	100 mL of 10× stock 500 mL of water 200 mL of methanol Add water to 1000 mL.

[a] Dithiothreitol.

[b] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Table 5. Common Western Blot Buffer Formulations *(Continued)*

Buffer	Content
TBS 10×	24.23 g of Tris base 80.06 g of NaCl Mix in 800 mL of ultrapure water. Adjust with pure HCl to a pH of 7.6. Add water to 1000 mL.
TBS-T	100 mL of TBS 10× 900 mL of water 1 mL of polysorbate 20
8.5 M urea stock	510 g of urea Add water to 1000 mL.
Sample buffer 2D electrophoresis	47 mL of 8.5 M urea stock 385 mg of tributyl phosphine (TBP) 2 g of CHAPS[b] 25 mg of bromophenol blue 1% carrier ampholytes of choice

[a] Dithiothreitol.
[b] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Methanol is necessary only if analysts use nitrocellulose. If they use PVDF, they can remove methanol from the transfer buffer and need only to activate the PVDF before they assemble the gel and membrane sandwich.

In semidry transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between the cathode and anode. During wet transfer the membrane should be closest to the positive electrode, and the gel should be closest to the negative electrode. The composition of the transfer buffer is not necessarily the same as the migration or running buffer. Analysts should consult the apparatus manufacturer's protocol, and it is common to add both SDS and methanol. The balance of SDS and methanol in the transfer buffer, the proteins' molecular weights, and the gel percentage can affect transfer efficiency for both wet and semidry transfers.

BLOCKING

Blocking the membrane prevents nonspecific background binding of the primary and secondary antibodies to the membrane. Traditionally, one of two blocking solutions is used: nonfat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phosphoproteins. To prepare a 5% milk or BSA solution, weigh 5 g/100 mL of Tris-buffered saline containing polysorbate 20 buffer (TBS-T; see *Table 5*). Mix well, and filter. Failure to filter can lead to spotting in which tiny dark grains contaminate the blot during development. Incubate at 4° for 1 h with gentle shaking. Rinse in TBS-T after the incubation.

PRIMARY ANTIBODY AND INCUBATION BUFFER

Dilute the antibody with blocking buffer at a proper dilution (1:100–1:3000, depending on antibody titer), and optimize the dilution according to the results. Too much antibody can result in nonspecific bands.

INCUBATION TIME

Incubation time can vary between a few hours and overnight, and depends on the binding affinity of the antibody for the protein and the abundance of protein. A more dilute antibody with a prolonged incubation may improve specific binding.

INCUBATION TEMPERATURE

It is best to incubate under cold temperatures. When analysts incubate in blocking buffer overnight, they should incubate at 4° to prevent contamination from bacterial growth, and should gently agitate the antibody solution to enable adequate homogeneous covering of the membrane.

SECONDARY ANTIBODY AND INCUBATION BUFFER

Handle the secondary antibody and incubation buffer as follows. Wash the membrane several times in TBS-T while agitating to remove residual primary antibody. Dilute the secondary antibody with TBS-T at the suggested dilution. Too much secondary antibody can result in nonspecific bands. Incubate the blot at room temperature for 1–2 h with gentle agitation. *Table 1* shows multiple options for secondary detection reagents and methods. More details are available in the *Immunoblot Data Analysis* section below.

General Chapters

Slot/Dot Blot

The procedure is similar to the procedure for 1D immunoblotting, but differs because protein samples are not separated electrophoretically but are spotted directly onto the membrane either manually or by use of a blotting unit (dot or slot blot format).

PROCEDURE USING MANUAL SPOTTING

Handle manual spotting as follows. Place a dry filter paper on a stack of dry paper towels. Place filter paper that is pre-wet with transfer buffer on top of the dry filter paper. Place a pre-wet membrane on top of the pre-wet filter paper. Samples are spotted onto the pre-wet membrane and are allowed to absorb into the membrane. After the sample is absorbed, place the membrane on a clean, dry filter paper to dry.

PROCEDURE USING A VACUUM-BLOTTING UNIT

Analysts typically use a vacuum-blotting unit as follows. Prepare a membrane, and place it in the blotting unit according to the manufacturer's instructions. Apply vacuum to the blotting unit to remove excess buffer. To improve solubility, dissolve the sample in a buffer, and if it is not clear, remove precipitates by centrifugation. If the sample is too viscous to pipet, then dilute it further with buffer. With the vacuum off, carefully pipet samples into the wells, and apply vacuum to the blotting unit. After all the samples have filtered through the membrane, turn off the vacuum, add buffer to each well to wash down the sides, and apply vacuum again. Remove the membrane, and proceed with immunoblotting.

2D Immunoblotting

SAMPLE PREPARATION

The compounds used to solubilize proteins must not increase the ionic strength of the solution. For example, a common sample solubilization solution is the following: 8 M urea, 50 mM dithiothreitol (DTT) or 2 mM tributyl phosphine (TBP), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, and 0.0002% bromophenol blue. The addition of carrier ampholytes enhances the solubility of proteins as they approach their isoelectric points. The use of ampholytes produces an approximately uniform conductivity across the pH gradient without affecting its shape, meaning that the concentration of carrier ampholytes should be optimized.

CHARGE SEPARATION

Several vendors produce and sell immobilized pH gradient (IPG) strips, or they can be made in-house according to ⟨1054⟩. The choice of IPG strips depends on the pI of the proteins of interest. The size of the IPG should match the size of the second-dimension gel. The amount of protein in each sample should be determined, and the amounts loaded on the IPG strips should be in the range of 10–300 µg, depending on the size of the IPG. The sample and standard should be loaded according to the manufacturer's instructions or according to ⟨1054⟩. Analysts then proceed with the isoelectric focusing by applying the electrical parameters described in ⟨1054⟩ or by the manufacturer.

MOLECULAR WEIGHT SEPARATION

After charge separation, analysts must equilibrate the strip in SDS-containing buffer before separation in the second dimension to determine molecular weight (as described previously in the *1D Immunoblotting* section). Analysts should position the strip directly on top of the gel, then secure the strip by overlaying it with 0.5%–1.0% agarose prepared in SDS–PAGE running buffer. To track the ion front in the second dimension, analysts can add bromophenol blue to the agarose.

IMMUNOBLOT DATA ANALYSIS

The presence or absence of bands usually is determined by comparison to a control (highly characterized antigens known or qualified to give a precise or expected response) of a type that is similar to the antigen being processed. Although analysts usually perform a qualitative comparison, bands or dots can be quantitated using a detection system (e.g., after incubating in a solution containing 4-CN peroxidase substrate; also see *Table 1*), and are compared to the control bands run in parallel (e.g., in the same gel).

Detection Options

ENHANCED CHEMILUMINESCENCE

Enhanced chemiluminescence is a popular method for detection in immunoblot analysis because it is highly sensitive (detection to pg or lower levels), and can be used to quantitate the relative concentration of the protein of interest. The method depends on incubation of the blot with a substrate that luminesces when exposed to the reporter on the secondary antibody. The light is detected using either photographic film or a charge-coupled device (CCD) camera. The image then is analyzed by densitometry to evaluate the relative amount of protein staining in terms of optical density. By using an appropriate set of molecular weight standards as markers, analysts can estimate molecular weight.

FLUORESCENCE DETECTION

Direct fluorescence can be used to detect proteins on blots. Direct fluorescence is simple, rapid, sensitive, and has a greater linear range than enhanced chemiluminescent detection. The advantage of direct fluorescence is the ability to detect many different fluorescent signals. This analysis avoids the need to reprobe the blot. Compared to enhanced chemiluminescence, fluorescence methods are easier to visualize and quantitate on CCD or laser-scanning imaging systems. Some data-acquisition systems permit extending the time of data acquisition to optimize signal-to-noise levels. Fluorescence-labeled blots that can be re-examined are useful for this purpose.

Enhanced chemifluorescence (ECF) is another common fluorescence method. ECF uses secondary antibodies conjugated to either HRP or AP. The enzyme-conjugated antibodies react with specific substrates that produce fluorescence after enzymatic cleavage. Analysts visualize the resulting signals using UV epi-illumination and capture digital images. An ECF signal has a greater linear range than traditional enhanced chemiluminescence. For example, direct fluorescence has a limit of detection in the pg range, and also has about 2 logs of linear dynamic range.

Quantum dots also are an alternative to detect proteins in immunoblot analysis. Quantum dots are a type of probe that can be conjugated to antibodies simultaneously or sequentially to detect multiply labeled antigens, without the need for blot stripping. Similarly, near-infrared (NIR) fluorophore–linked antibody is a method for antibody detection whereby light produced from the excitation of a fluorescent dye is measured in a static state. Light measured in a static state allows more precise and accurate detection than light measured in a dynamic state (e.g., chemiluminescence).

RADIOACTIVE DETECTION

Proteins also can be detected by labeling an antigen with a radioactive isotope (e.g., iodine). On the one hand, this method has the advantage that the radioactivity in a band is easy to quantitate by means of time exposure to film and densitometry, or by directly excising the band from the membrane and counting using a scintillation counter. On the other hand, radioactivity also introduces the disadvantage of safety because analysts must manage radioactive material, and analytical laboratories must have a program to control and monitor waste management and individual exposure.

Immunoblot Quantitation

NONELECTROPHORETIC QUANTITATION

The quantitation of a specific protein is achieved when the blot procedure is properly optimized and generates a linear response range over a particular time frame. Immunoblot quantitation includes several elements: adequate antigen and antibody concentrations and purity, antibody specificity, blocking conditions, sufficient washes, and the duration and intensity of the signals. Once the exposures are captured on a film or electronically under optimized conditions, analysts use densitometric methods to quantitate results by comparing a specific protein on the blot and on the standard. Analysts can correct results for background by including a negative control.

The intensity of the bands depends on the amount of protein. Different commercial software packages are available for image analysis of bands on a film. Alternatively, digital imaging systems containing CCD cameras usually include software designed to perform data analysis.

ELECTROPHORETIC QUANTITATION

Proteins of various molecular weights are identified by the extrapolation of plots of relative mobilities of prestained proteins of known molecular weight and can be compared to the positive control. Positive controls are trended to determine the limit range of the densitometry results compared to the nominal concentration results. Independent of the detection method, the following criteria must be met for a valid Western blot result.

- Ensure adequate development by minimizing membrane overexposure and visualizing staining controls.
- The prestained molecular weight markers must be visible and must cover the anticipated range.

- The band(s) should have the appropriate location and intensity for the standard, the control, and the protein of interest.
- There should be no blot or staining artifacts that obscure the visualization and interpretation of bands.

METHOD VALIDATION

As outlined by ICH Guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology (effective November 2005) and *USP* general chapter *Validation of Compendial Procedures* ⟨1225⟩, a qualitative assay such as the slot/dot blot requires validation of specificity. Specificity is the ability to detect the analyte in the presence of other components. For validation, it should be shown that the particular steps of the slot/dot blot method can detect the antigen when present and do not report false positive results when the antigen is absent. In addition, demonstration of the specificity of the antigen-specific antibodies is part of the specificity evaluation.

USP general chapter ⟨1225⟩ provides guidelines for the validation of analytical procedures, and analysts should consider this resource when they validate immunoblot methods. All methods require a demonstration of the specificity of the antibody to the antigen and the lack of recognition of other proteins and reagents in the matrix. Identity tests require only specificity. Limit tests require specificity and LOD. A sensitivity control incorporated into each test can show that the LOQ is met on each determination to account for potential changes in the sensitivity of the method. A quantitative test requires all ICH validation parameters, including robustness testing.

Demonstration of electrophoretic immunoblot specificity should include the following: stained gels to show protein separation, stained blots to show adequate protein transfer to the membrane, blots with control samples to show the specificity of the conjugate to the primary antibody, and blots that show the binding of the antibody to the appropriate antigen. Method validation also can identify the need for control membranes for each assay, as well as protein sensitivity controls as measures of system suitability.

⟨1105⟩ IMMUNOLOGICAL TEST METHODS—SURFACE PLASMON RESONANCE

Introduction

Surface plasmon resonance (SPR) optical detection is a useful method for the label-free assays (procedures) that study biomolecular interactions. Commercially available SPR biosensors that incorporate these assays can collect real-time, information-rich data from binding events. These data can be used widely from basic research to drug discovery and development to manufacturing and quality control (QC). SPR can characterize binding events with samples ranging from proteins, nucleic acids, and small molecules to complex mixtures, lipid vesicles, viruses, bacteria, and eukaryotic cells. Typical quality and safety attributes addressed with SPR analysis include:

- Interaction specificity
- Interaction affinity
- Kinetic binding parameters
- Thermodynamic parameters
- Biologically active concentration of an analyte

This chapter provides an overview of the physics underlying SPR and common instrument configurations, as well as the range of molecules that can be studied and general considerations for experimental design as determined by the assay objective.

Overview

History

The physical principles of SPR were first explained in the early 1900s, starting with a description of the uneven distribution of light in a diffraction grating spectrum caused by the excitation of surface plasmon waves. A landmark series of experiments showed the optical excitation of surface plasmons under conditions of total internal reflection and fostered detailed studies of the application of SPR for chemical and biological sensing. Since then, SPR's potential for characterizing thin films and monitoring interactions at metal interfaces has been recognized, and significant research and development have yielded instruments that can quantitatively evaluate the binding interactions of small and large molecules.

Physics

SPR is an optical phenomenon that occurs when a thin conducting film is placed between two media that have different refractive indices. In many commercially available instruments, the two media are glass and the sample solution, and the conducting film is preferentially a gold layer applied to the glass, although other conducting metals such as silver have been used. The glass–metal component comprises a solid support that is often referred to as a *sensor*.

Light applied to the glass under conditions of total internal reflection produces an electromagnetic component that is called an *evanescent wave*. The evanescent wave penetrates the medium of lower refractive index (typically the sample solution) without losing net energy. The amplitude of the evanescent wave decays exponentially with distance from the surface, roughly one-half of the wavelength of the incident light (e.g., for a light source of 760 nm the evanescent wave penetrates approximately 300 nm).

For a specific combination of wavelength and angle of incident light, electron charge density waves called *plasmons* are excited in the gold film. As energy is absorbed via the evanescent wave, a decrease in the intensity of the reflected light at a specific angle (the SPR angle) is observed. Analysts can conduct an SPR experiment by fixing the wavelength and varying the angle of incident light.

An increase in mass at the sensor surface caused by a binding interaction between two or more molecules causes a change in the local refractive index (RI) that gives rise to an SPR response, which is observed as a shift in the SPR angle. By monitoring the shift in the SPR angle as a function of time, an analyst can generate a *sensorgram* (*Figure 1*). The change in RI is very similar for different proteins, so the SPR measurement depends primarily on the mass change at the sensor surface and is relatively independent of the nature of the molecules being measured.

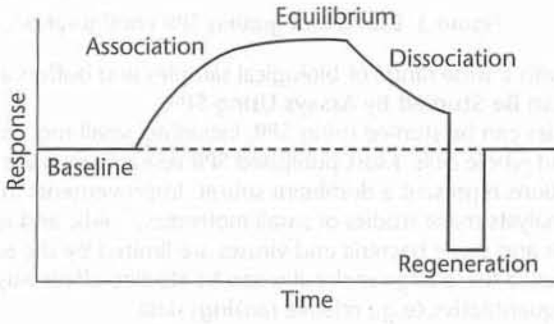


Figure 1. Representative sensorgram.

Instruments

The main components of commercially available SPR instruments are (1) a light source, typically a high-efficiency light-emitting diode, (2) an optical detector such as a diode-array or charge-coupled device camera, (3) a solid support containing the conducting film and some means for attaching molecules, (4) a sample delivery system, frequently a microfluidic device capable of delivering samples using single serial or parallel injections via single or multiple needles, and (5) a computer with appropriate software for instrument control, data collection, and analysis.

Prism-based and diffraction-grating instrument systems are commercially available. Most prism-based systems follow the Kretschmann configuration (*Figure 2*). The light is focused onto the sensor surface (away from the samples) via a prism with a refractive index matching that of the surface. In this configuration the incident light does not penetrate the sample solution, which permits SPR measurements for heterogeneous, turbid, or opaque samples. In systems that utilize a diffraction grating (*Figure 3*) the analyte solution is placed over a plastic surface on which a metal has been deposited. The plastic acts as an attenuated total internal reflection prism in which light reflected from the grating is reflected many times back to the grating surface. In this configuration light passes through the analyte sample solution, and thus turbid or opaque samples are not suitable for measurement. The diffraction grating does permit sampling of a larger surface area and is applicable for SPR measurements of arrays.

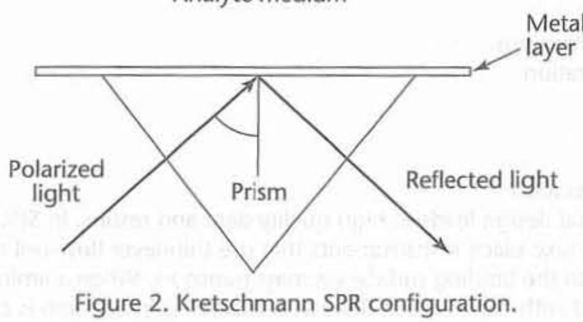


Figure 2. Kretschmann SPR configuration.

General Chapters

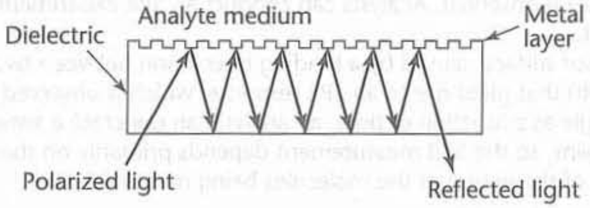


Figure 3. Diffraction grating SPR configuration.

The instruments are compatible with a wide range of biological samples and buffers as well as some organic solvents.

Biomolecular Interactions That Can Be Studied By Assays Using SPR

A diverse range of biological entities can be studied using SPR, including small molecules (<100 Da), proteins, nucleic acids, lipids, bacteria, viruses, and whole cells. Most published SPR research involves protein–protein interactions, of which antibody–antigen interactions represent a dominant subset. Improvements in instrument sensitivity and experimental protocols have helped analysts make studies of small molecules, lipids, and nucleic acids. Protein interactions with larger entities such as whole cells and some bacteria and viruses are limited by the exponential decay of the evanescent wave as described above. In practice these large molecules can be studied effectively, but the information obtained may be limited to qualitative or semiquantitative (e.g., relative ranking) data.

Assay Types

Several types of SPR assays are useful, including binding specificity, concentration analysis, kinetics and affinity analysis, and thermodynamics. Each assay type generates unique information that is helpful for profiling biomolecules.

SPR is also suitable for use in qualitative studies to confirm the specificity of interactions. Analysts can monitor a number of sequential binding events because each individual event yields a mass increase on the sensor chip surface, and all stages in the binding process are monitored. Examples include epitope mapping, antibody isotyping, and immunogenicity measurements.

Most chemical and spectroscopic methods used to quantify proteins (1) measure total protein content, (2) do not distinguish active from inactive molecules, and (3) cannot be used in conjunction with unpurified samples. Because SPR is a noninvasive method (no light penetrates the sample), it can measure small amounts of analyte molecules from complex matrices such as food products, serum or plasma, and cell extracts. Direct or indirect (inhibition or competitive) formats for measuring concentration are possible. SPR biosensors are uniquely suited for measurement of kinetic association and dissociation rate constants from real-time measurement of binding interactions. Affinity can be derived either from interactions that have reached equilibrium or from the ratio of the dissociation and association rate constants. The typical working range for affinity measurements is pM to high µM concentrations. Association rate constants that can be measured typically range from 10^3 to 10^7 $M^{-1}s^{-1}$ and dissociation rate constants from 10^{-5} to 0.5 s^{-1}. By studying temperature dependence of rate and affinity constants, analysts can determine thermodynamic parameters for a binding interaction. Not only can the equilibrium values for changes in enthalpy (ΔH) and entropy (ΔS) associated with complex formation be determined, but transition state energetics can also be evaluated. Subsequent sections of this chapter address the specific details for these different assay types.

The SPR Assay

The typical SPR assay involves five steps:

1. Sample and buffer preparation
2. Surface preparation
3. Analyte binding
4. Surface regeneration
5. Data analysis and interpretation

Careful attention to experimental design leads to high-quality data and results. In SPR experiments, mass transport is essential for binding interactions to take place in instruments that use thin-layer flow-cell systems. Analyte molecules are transferred from the bulk solution to the binding surface via mass transport. When a limitation for binding occurs as a result of fast binding kinetics combined with high surface density, the binding interaction is considered mass-transport limited. In this case, the binding kinetics and complex formation are influenced by the availability of analyte molecules. The advantages and disadvantages of mass-transport–limited binding are discussed later in the application examples.

Sample and Buffer Preparation

Both purified and crude samples can be analyzed in a variety of matrices including serum, plasma, cell supernatants, and lysates. Crude samples containing particulates (e.g., cell debris or precipitates) may require clarification in order to help minimize unwanted binding. A short spin (30–60 s) in a benchtop centrifuge or filtration (0.22–1.0 µm) using low–protein-binding filters is recommended. The concentration range for evaluation depends on the experimental objective (yes/no binding, concentration, or kinetic/affinity analysis) as well as the binding affinity of the interacting molecules. In general, sample concentrations an order of magnitude below the equilibrium dissociation constant (K_D), can be detected

by SPR, but determination of an exact concentration is influenced by the analyte size (large vs. small molecules), binding specificity, and overall biological activity of the samples.

Most biological buffers and several organic solvents can be used in SPR experiments. The addition of salts and detergents to buffer solutions frequently can stabilize biomolecules. High-quality grade (e.g., molecular biology grade or higher) buffer components should be used. To simplify experiments, analysts should add only components that are absolutely required for biological activity or function. Buffers should be filtered and degassed before use.

Surface Preparation

Surface preparation involves the attachment of one of the binding partners to a solid support (surface). This process is frequently referred to as *immobilization*, and the resulting surface with the attached biomolecule is the sensor for the experiment. The choices of binding partner, solid support, and immobilization method are influenced by (1) the nature and demands of the application or experimental objective; (2) the availability of surfaces with different properties (e.g., charge density, hydrophobicity, or hydrophilicity); (3) the characteristics and supply of biomolecule to be used for immobilization; and, most importantly, that (4) biological activity be maintained and binding sites be available to interacting partners. Depending on the experimental objective, homogeneous or orientation-specific attachment of biomolecules also may be desired. The two main categories of immobilization methods are (1) direct immobilization, in which the molecule is covalently attached to the surface, and (2) indirect or capture immobilization, which takes advantage of tags or native groups on the protein or biomolecules (*Table 1*).

Table 1. Surface Preparation Techniques

Chemistry	Immobilization Method	Biomolecules	Comments
Amine	Direct	Proteins, peptides	Amino terminus, Lys residues
Thiol—native	Direct	Proteins, peptides	Native Cys residue
Thiol—added	Direct	Proteins, peptides	Carboxyl groups derivatized
Aldehyde	Direct	Glycoproteins	*Cis–diol* required
Biotin capture	Indirect	Biotinylated peptides, nucleic acids, proteins	Stable, irreversible capture
Affinity tags	Indirect	Proteins, peptides	His, Glutathione *S*-transferase (GST), etc.
Protein A, Protein G	Indirect	Antibodies, IgG-tagged molecules	IgG species–dependent
Protein A, Protein G	Indirect	Biomolecules specific to the capturing antibody	Mono- or polyclonal antibodies may be suitable—testing recommended
Hydrophobic adsorption, membrane capture	Indirect	Lipids, membranes, membrane-associated proteins	Monolayer or bilayer attachment possible

Direct Immobilization: For direct immobilization, several chemistries are available for attaching proteins or other biomolecules to the surface. The properties of the surface determine the specific sequence of steps and length of time required to prepare the surface. Many commercially available surfaces have a biologically compatible layer (e.g., a hydrogel) that contains functional groups such as carboxyl that can be used for immobilization. To ensure binding specificity, the purity of the biomolecule that is attached to the surface should be 95% or greater and the required concentration should range from 1 to 1000 µg/mL. Direct immobilization chemistries frequently result in heterogeneous surfaces because of random orientation of biomolecules on the surface.

Immobilization via free primary amine groups such as lysine residues in proteins or the amino terminus of proteins or peptides is one of the most generally applicable covalent chemistries for attaching proteins to a surface. Carboxyl groups on the surface are converted to reactive esters using a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) or sulfo-NHS (sNHS). The protein or biomolecule is applied in high concentrations (mg/mL) to maximize the efficiency of amine coupling. Finally, free esters are blocked with ethanolamine. The contact time with the surface, the protein concentration, or the EDC/NHS concentration can be varied to adjust the immobilization level.

When amine coupling interferes with the binding site, the biomolecule can be attached using alternative coupling chemistries or a high-affinity capture approach. For example, for biomolecules with free thiol groups (typically cysteine residues), a disulfide group is introduced by treating the surface with NHS and EDC to attach 2-(2-pyridinyldithio)ethaneamine (PDEA). Adding the biomolecule to the surface results in thiol–disulfide exchange, and excess PDEA groups are inactivated with cysteine–HCl. If the biomolecule lacks a free thiol group, a reactive disulfide (PDEA) can be linked to carboxyl groups. Subsequently the pyridyldisulfide groups can be attached to thiol groups on the surface that have been derivatized via injection of NHS and EDC, followed by cystamine, then reduction with dithioerythritol (DTE) or dithiothreitol (DTT). Attachment of maleimide groups to the surface makes possible an alternative form of immobilization via thiol groups in which a stable thioether bond is formed. Surfaces prepared using this method have the capacity to withstand basic pH (> 9.5) and reducing agents such as β-mercaptoethanol and dithiothreitol. Several heterobifunctional reagents are available commercially for introduction of reactive maleimido groups to the surface, including sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester), sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate), GMBS [*N*-(γ-maleimidobutyrloxy)sulfosuccinimide ester], EMCH [*N*-(ε-maleimidocaproic acid)-hydrazide] or BMPH [*N*-(β-maleimidopropionic acid)-hydrazide]. For biomolecules containing either native ald-

General Chapters

hehyde groups or *cis–diols*, which may be converted into aldehydes by mild oxidation, surface attachment via a hydrazone bond is an option. Hydrazide groups on the sensor surface react with aldehyde groups on the biomolecule to form a stable bond. Immobilization via aldehyde groups is most useful for glycoconjugates, glycoproteins, and polysaccharides.

Indirect (High-affinity Capture) Immobilization: Indirect or high-affinity capture immobilization approaches use tags commonly used for protein purification. This technique exploits the high-affinity capture of the biomolecule by a capturing molecule that has been immobilized covalently using one of the techniques described above. The requirement for biomolecular purity is less stringent for indirect versus direct immobilization because the capturing step for the biomolecule can also provide purification. Indirect immobilization frequently yields a homogenous surface because all biomolecules are oriented similarly via the tag. The affinity between the biomolecule and its capturing agent should be sufficiently high to ensure little or no dissociation from the surface for the duration of an analysis cycle. Monoclonal antibodies are frequently used as capture molecules. For example anti-GST antibodies can be attached to the sensor chip surface via amine chemistry in order to capture GST-tagged molecules. Protein A, Protein G, and anti-IgG antibodies are useful capturing molecules for use with antibodies.

The high-affinity interaction between streptavidin or related molecules and biotin ($K_D \approx 10^{-15}$ M) makes it a useful system for the capture of biotinylated molecules (e.g., proteins, peptides, nucleic acids, membranes, and liposomes). Frequently, the biotin-binding protein is attached to the surface using primary amine groups. Because of the high affinity of the interaction, biotinylated molecules are considered permanently immobilized, and in contrast to most other capture approaches biotinylated molecules cannot be removed without damaging the surface. Histidine (His)-tagged recombinant proteins can be captured via nickel–NTA chemistry or covalently immobilized anti-His antibodies.

Lipids and membrane-associated proteins can be captured to the surface as either a lipid monolayer or bilayer. Lipids from micelles or liposomes adsorb to a hydrophobic surface, creating a lipid monolayer with the hydrophobic lipid tails oriented toward the solid support and the hydrophilic heads towards the aqueous sample. This approach provides a stable environment for proteins associated with a membrane surface or partially inserted into the membrane, but it is not ideal for transmembrane proteins because the resulting surface presents only half the membrane structure for binding interactions. Intact membrane structures (lipid bilayers) with associated or incorporated proteins can be captured by preparing liposomes with a specific antigenic component or with biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin, respectively.

Additional Considerations: Once the biomolecule has been attached to the sensor using either a direct or indirect immobilization approach, analysts should assess the baseline stability of the newly created surface. If the baseline is decreasing (downward drift), the most likely cause is the presence of unattached biomolecules, possibly because of self-association or aggregation. If the baseline is increasing (upward drift) refolding or re-orientation may be causing the change. In either case, the newly created surface should be conditioned before use by one or more of the following: (1) multiple injections of biologically compatible buffer; (2) washing the surface with buffer at a fast flow rate; (3) multiple injections of either high ionic strength (e.g., 1 M NaCl) or detergent (e.g., 20 mM CHAPS or 0.05% Polysorbate 20 (P20)) solutions; or (4) repeated analyte binding and regeneration injections. NOTE: recommendations (3) and (4) should be used only if the activity of the biomolecule in the presence of these reagents has been evaluated previously.

Large baseline drifts caused by low-affinity capture may be overcome by using EDC/NHS as a cross-linking step, but this may compromise biomolecule activity if active sites of the biomolecule are involved in the cross-linkages. The effect of cross-linking on biomolecule activity must be tested empirically for each biomolecule–analyte system. In general, cross-linking should be as brief as possible: 15 s is often sufficient to achieve acceptable baseline stability without compromising biomolecule activity.

How Much to Immobilize: The amount of biomolecule to immobilize depends on the experimental objective. Equations 1 and 2 are useful for calculating the appropriate surface density:

$$R_{max} = (MW_A/MW_L) \times R_L \times S_m \quad \text{[Equation 1]}$$

$$R_L = R_{max} \times (1/S_m) \times (MW_L/MW_A) \quad \text{[Equation 2]}$$

R_{max} = theoretical maximum binding response (assuming a surface that is 100% active and 100% bound with analyte)
R_L = response of the immobilized molecule
MW_A = molecular weight of the analyte
MW_L = molecular weight of the immobilized molecule
S_m = molar binding stoichiometry

For kinetic experiments, a low density of immobilized molecule is preferred in order to avoid steric hindrance, aggregation, and/or mass-transport–limited binding. Low density is defined as R_L that limits R_{max} to 5–50 response units. For other applications, e.g., concentration analysis where mass-transport–limited binding is desired, R_{max} can be 100–200 times higher than for kinetic experiments provided that steric hindrance or aggregation are not induced. Specific recommendations for immobilization density are included in the application examples for this chapter.

Analyte Binding

Samples that will be evaluated for binding using SPR do not require the same purity as biomolecules intended for direct immobilization onto the surface. Because the light source does not penetrate the sample, turbid or opaque samples can be analyzed by SPR. Whenever practical, samples should be clarified according to the recommendations given under *Sample and Buffer Preparation*, and buffer additives should be minimized, including only the amount required for biological activity.

Differences between the refractive index of the bulk and sample buffers give rise to a response. The use of control surfaces and samples aids in demonstrating binding specificity for the molecules in SPR. For direct immobilization methods, suitable control surfaces can be (1) the sensor surface without any modification or biomolecule attached, (2) a surface that has been chemically treated in the same manner as the surface containing the biomolecule, or (3) a related but known nonbinding biomolecule. For surfaces prepared using indirect (capture) immobilization the capturing molecule in the absence of the tagged binding partner should be used as the control surface. The difference in response between the control and active surfaces gives an initial indication of the binding specificity. Concentration-dependent responses and inhibition of binding by incubating the sample with the biomolecule on the surface can further establish the binding specificity.

If nonspecific or unwanted binding is observed, analysts should determine the source. Frequently changes in pH or ionic strength of the buffers used in the experiment will reduce or eliminate the unwanted binding. Additional suggestions for reducing nonspecific binding are summarized in *Table 2*.

Table 2. Suggested Actions for Reducing Nonspecific Binding

Category	Action
Experimental Design	1. Optimize running buffers: (a) increase salt (150 to 500 mM) (b) add detergent (0.001% to 0.05%) (c) match composition of sample and running buffers 2. Change ligand immobilization method 3. Evaluate ligand quality 4. Increase or decrease temperature in detection chamber
Choice of Surface	1. Change properties of sensor surface: (a) reduce electrostatic interactions (b) evaluate hydrophobic vs. hydrophilic character of surface (c) consider alternative ligand to use for control surface 2. Pre-immobilize amino—PEG 3. Change blocking molecule (e.g., ethylenediamine)
Additions to Sample	1. Add nonspecific binding reducer to sample: (a) increase ionic strength of running and sample buffers (e.g. 150 to 500 mM NaCl) (b) add detergent to running and sample buffers (e.g. 0.001% to 0.05% surfactant P20) (c) add soluble carboxymethyl dextran (1–10 mg/mL, for dextran-based surfaces only) 2. Simplify sample buffer—include only components required for biological activity 3. Evaluate analyte quality

Equations 1 and 2 are also useful for assessing surface activity. The higher the binding response, the more active the surface is unless the observed binding response exceeds the calculated R_{max} value. In this case, the molar binding stoichiometry is incorrect, the analyte molecule is aggregated, or the analyte is binding nonspecifically to the surface. Binding responses that are low (< 10% of R_{max}) suggest that the analyte concentration selected for the experiment is too low or that the surface activity of the immobilized molecule is low. In the former case, increasing the analyte concentration should increase the binding response, and in the latter situation using a different immobilization method may be helpful.

Surface Regeneration

Surface regeneration refers to the process of removing bound analyte from the surface in order to reuse the surface for subsequent binding interactions. In some instances, complex dissociation is fast and bound analyte is simply washed away with buffer, so regeneration is not needed. Alternatively, the instrument configuration may allow multiple samples to be injected either sequentially or in parallel across several immobilized surfaces simultaneously, thereby limiting the need for regeneration. Inadequate surface regeneration may affect the reproducibility of an assay and negatively affect the overall quality of the resulting data. To identify the correct conditions, analysts should consider the nature of the specific interaction and the experimental objective. For example, a slight baseline drift will not affect the results when a simple yes/no answer is sought, but in concentration determination or kinetic studies, optimization of the regeneration step is critical.

Most biochemical interactions involve non-covalent bonds such as hydrogen, electrostatic, van der Waals, and hydrophobic bonds. Because the combination of physical forces responsible for binding and the regeneration conditions critical for not causing irreversible conformational changes are unknown for most interactions, the final conditions must be evaluated empirically.

The ideal condition for regeneration dissociates all the bound material without affecting the biological properties of the immobilized biomolecule. An incomplete regeneration or too stringent conditions may result in decreased analyte bind-

General Chapters

ing capacity in subsequent cycles because of either blocking of binding sites by nondissociated analyte or partial denaturation of the biomolecule. Regeneration buffers and solutions can be divided into different classes by the effect they have on the interaction. Any combination of buffers can be used.

The major classes of regeneration buffers are: acidic, basic, ionic/chaotropic, detergent, hydrophobic/nonpolar, and chelating (see *Table 3* for examples of each class). Analysts should start with mild conditions, moving progressively to more harsh conditions. In many cases, especially when one is working with antibodies, change in pH is the most effective method of regenerating the surface. Contact time with the surface is important for efficient regeneration. When analysts use pH change, the contact times should be short, one-half to 2 min. When analysts use high ionic strength or chaotropes, longer contact times of 2–4 min are usually effective.

Table 3. Examples of Regeneration Solutions

Acid	Base	Ionic/ Chaotropic	Detergent	Hydrophobic/ Nonpolar	Chelating
1–100 mM HCl	1–100 mM NaOH	0.5–5 M NaCl	0.02%–0.5% SDS	25%–100% ethylene glycol	10–20 mM EDTA or EGTA
10–100 mM glycine, pH 1.3–3.0	10–100 mM glycine, pH 9.0–10.0	1–4 M $MgCl_2$	40 mM octyleneglycol + 20 mM CHAPS	5%–50% DMSO	10–200 mM imidazole
10–100 mM phosphoric acid	1 M ethanolamine HCl, pH 9.0 or above	1 M KSCN	40 mM octylglucoside, 40 mM octylglucoside	1%–10% acetonitrile	
0.1% TFA	100 mM sodium carbonate + 1 M NaCl, pH 9–11	2–6 M guanidine HCl			
100 mM Formic acid	20–100 mM NaOH containing 0.5% surfactant P20 or 0.05% SDS				

The purpose of optimizing the regeneration conditions is to find the mildest possible regeneration solution that completely dissociates the complex. Analysts should maintain a constant level of activity over the binding–regeneration cycles even if the baseline changes a little. Repeated cycles of analyte binding followed by regeneration of the surface will provide insight into the overall performance of the surface. Ideally the surface performance should be evaluated for the same number of cycles that will be used during the SPR experiment.

The surface must be monitored for signs of accumulation and also degradation of the immobilized ligand (*Figure 4*). This can be accomplished by monitoring both the baseline at the beginning of each injection cycle and binding signal (slope or bound response) of a quality control sample. Appropriately defined acceptance criteria for system suitability such as baseline drift and quality control performance help to monitor the integrity of the immobilized ligand on the surface.

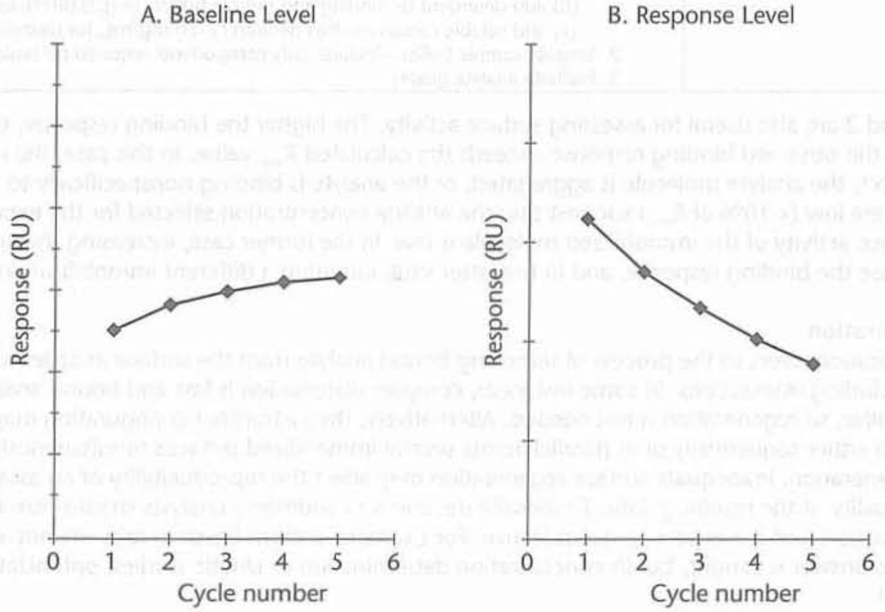


Figure 4. Evaluating surface performance (A) accumulation on surface and (B) degradation of immobilized ligand.

If the binding response is slowly decreasing, there are two possible explanations:

1. If the baseline of the raw data sensorgram remains constant but the binding response still decreases, the regeneration conditions cause an irreversible change to the biomolecule that decreases the binding capacity of the surface, which in turn decreases the amount of analyte that can be bound on the surface. Analysts can decrease the

strength of the regeneration solution slightly or can change to another regeneration solution of equal strength within the same class.

2. If the baseline increases, the accumulation of analyte causes a binding-capacity decrease on the surface, which in turn decreases the amount of analyte that can be bound to the surface. Analysts can increase the strength of the regeneration solution slightly or can change to a regeneration solution of equal strength within the same class. There may be a difference between regeneration solutions in their ability to solubilize the analyte.

Once a suitable regeneration solution has been determined, it should be tested in a series of analyte binding and regeneration cycles. Because the binding activity of the surface typically decreases with time and/or use, analysts must empirically determine the binding response threshold and consequent number of cycles for surface use. Examples for determining binding threshold and number of cycles are presented below (see Applications 1–3).

In some cases the baseline will drop or rise and/or the binding capacity will decrease somewhat in the first few injections before it stabilizes. This is caused by either the dissociation of electrostatically bound biomolecules from the surface (depending on surface characteristics) or to binding to a high-affinity non-regenerable fraction of the surface. For this reason, each newly immobilized surface should be conditioned with repeated analyte binding and regeneration cycles before collection of quantitative data. Alternative immobilization methods, such as a different chemistry or indirect capture, should be evaluated when the immobilized biomolecule is difficult to regenerate.

Data Analysis and Interpretation

Analysis and interpretation of the data are specific to the experimental objective. Several data analysis programs exist to aid in the calculation of kinetics and affinity constants from SPR data. The validity and quality of the results are linked directly to experimental design. The fitting process is purely mathematical, without regard to the biological significance of the values obtained.

Data Analysis Algorithm: Global analysis seeks a single set of kinetic rate constants for all of the analyte concentrations used in the experiment. Using a data-fitting algorithm such as Marquardt-Levenberg the data analysis software begins an iterative process starting with an initial approximation to find the best set of parameters that produces agreement between the experimental data (sensorgram) and the calculated fit to the data. The iterative process continues until the difference between the experimental and calculated (theoretical) curves is minimized as measured by the sum of the squared residuals.

Preparing the Data for Analysis: Before conducting kinetic analysis, analysts should inspect the experimental data visually for anomalies or artifacts such as baseline disturbances or out-of-range data (often due to air bubbles) lasting for a predefined time period (e.g., 4–8 s). Outliers should be removed from the data set according to pre-established criteria. Nonessential data, such as capture or regeneration injections, should be removed from the sensorgram, and the data at each analyte concentration should be adjusted using the double-referencing procedure described below.

Before analysis the raw data should be processed in the following manner:

- Align the injection start to zero seconds for all concentrations and buffer injections for both the reference and active surfaces.
- Align the baseline to zero response for all sensorgrams.
- Subtract the reference surface sensorgram from the active surface sensorgram in order to create a corrected data set.
- Subtract the corrected buffer sensorgram from the sensorgrams at different concentrations in order to create a double-referenced data set.

The double-referencing procedure removes systematic errors (e.g., instrument noise) and low levels (less than 5% of total binding response) of nonspecific binding. It should not be used to correct for significant nonspecific binding events because this can lead to erroneous measurements.

When analyzing the data for kinetic information, analysts use the association (injection) and dissociation (buffer flow) phases for all of the concentrations in the series. For steady-state affinity analysis, the response at equilibrium R_{eq} (data plateau or no change in response vs. time) is measured for each sensorgram to create a binding isotherm with R_{eq} vs concentration. This isotherm is analyzed using the equations described below.

Kinetics and Steady-state Affinity Models: The Langmuir kinetic model assumes a 1:1 interaction between the binding partners so that

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

The association and dissociation rate constants are defined below:

$$\frac{d[AB]}{dt} = k_a \times [A] \times [B]$$

$$-\frac{d[AB]}{dt} = k_d \times [AB]$$

Combining these two equations and defining $[B_{free}] = [B_{tot} - AB]$, the net rate expression is

$$d[AB]/dt = k_a \times [A_{free}]\,[B_{tot} - AB] - k_d \times [AB]$$

which can be translated into terms from the SPR experiment as follows:

$$dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$$

where R is the binding response at any point along the sensorgram and C is the known analyte concentration. Using global analysis as described above, k_a, k_d, and R_{max} are calculated from the experimental data using the rate equations shown below:

Association: $dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$

Dissociation: $dR/dt = -k_d \times R$

Because the concentration of analyte is zero during dissociation, the rate equation for dissociation depends only on the response, R, and the dissociation rate constant, k_d.

Application of the equilibrium condition where the complex formation (association) equals complex decay (dissociation)

$$k_a \times [A] \times [B] = k_d \times [AB]$$

yields the following equation for the equilibrium dissociation constant

$$K_D = \frac{[A] \times [B]}{[AB]} = \frac{C(R_{max} - R_{eq})}{R_{eq}}$$

where R_{eq} is the binding response at equilibrium that is measured in the experiment for a given analyte concentration and C, K_D, and R_{max} are calculated using global analysis.

Kinetics binding models can be used to describe non-1:1 interactions, e.g., bivalent interactions that occur if an antibody is used as the analyte, heterogeneity in binding partners, conformational change, or more complex interactions such as cooperative binding. SPR analysts are cautioned against using more complex models to assess data unless experimental design has been confirmed.

Assessing the Fit: The quality and validity of the fit to the kinetic data can be assessed by (1) visual inspection of the agreement between the experimental and calculated curves, (2) the size and the shape of residual plots, (3) the biological relevance of the results, and (4) statistical parameters such as χ^2 (average of squared residuals), and standard error (SE), T-value or U (uniqueness) factor. The best parameter fit to the experimental data should be superimposed on the curve for each concentration in the experiment. The residual plot visualizes the difference between the calculated and experimental data. The shape of the residuals should be random without trending (waviness or curving up or down). The height of the residual plot should reflect the instrument noise. Further, χ^2 should be minimized for a good fit with values that depend on the instrument noise, number of data points, and overall binding response. The parameter values should be considered for biological and experimental relevance. For example, is the calculated k_a value slow when the interaction is known to be fast, or is the calculated R_{max} value higher than the value that was calculated using Equations 1 and 2. Parameter significance is evaluated based on standard error, T-values, and U factor. Parameters that are significant cannot be changed without affecting the quality of the fit. All of the criteria should be within acceptable limits.

A similar set of criteria can be used for assessing the fit to steady-state affinity data, but because there are fewer data points (6–12 total, depending on the number of concentrations used), the statistical parameters and residual plots are less predictive of fit quality. Visual inspection of the agreement between the experimental and calculated binding isotherms and the parameter relevance are good tools to use for assessing the fit. Additionally, according to the relationship between concentration, K_D, and R_{max}, when concentration equals the K_D for the interaction R equals 50% of R_{max}. Confirming that the analyzed data follow this relationship provides another way to check the validity of the calculated result. When it is practical to calculate the K_D using both kinetic and steady-state analysis approaches, the K_D values should agree within experimental error.

Addressing a Lack of Fit: When the data do not fit or the parameter values do not make sense, often the problem can be resolved by a systematic approach that considers potential sources for deviations and tests each hypothesis. Items to consider include reagent purity, immobilization chemistry or surface density, analyte concentration errors, nonspecific binding, loss of ligand activity, or mass-transport–limited binding. Reviewing the raw (uncorrected) data helps determine the source of nonspecific binding or concentration errors. *Table 4* lists potential sources for deviations from 1:1 binding and recommended actions.

Table 4. Common Sources for Deviation from 1:1 Binding Kinetics

Source of Deviation	Recommended Action
Nonzero baseline before injection	• Normalize response to zero and reanalyze
Incorrect injection start and stop times or poorly defined injection start/stop	• Adjust injection start/stop • Remove sensorgram artifacts (e.g., injection or air bubble spikes)
Concentration input errors	• Verify concentration values and reanalyze
Bulk refractive index contribution too high	• Use double-referencing approach before analysis • Set RI = 0

Table 4. Common Sources for Deviation from 1:1 Binding Kinetics *(Continued)*

Source of Deviation	Recommended Action
Mass-transport–limited binding	• Vary flow rate (slow to fast) for a single concentration and overlay sensorgrams (should be identical for same association and dissociation time) • Include mass transport term, k_m, in fitting model • Reduce surface density
Nonspecific binding	• Change immobilization chemistry • Change sensor surface properties • Buffer additives—add or minimize • Reagent purity—repurify samples
Loss in binding partner activity	• Change immobilization chemistry • Change regeneration solution • Re-analyze data using local instead of global parameter fitting for R_{max}
Multi-valent binding interaction	• Immobilize multivalent binding partner

Recommendations for data analysis will be introduced in the subsequent sections of this chapter.

Application 1—Immunogenicity Assessment: SPR has emerged as a powerful technique for assessing immunogenicity of protein therapeutics. An advantage of this platform for detecting antibodies in serum (or plasma) samples is that it allows label-free detection based on mass accumulation in real time, which potentially allows detection of low-affinity antibodies of all classes and subclasses. This technology is useful both for screening assays (first-tier immunoassays that are used to detect the presence of antibodies capable of binding to a protein therapeutic) and also characterization assays. Characterization assays are useful for defining generated antibodies that bind to the protein and can include analysis of antibody concentration, isotype(s) represented, relative binding affinity, and binding specificity. A limitation is that SPR it is not appropriate for determination of the neutralizing capability of antibodies, which is best determined using cell-based biological assays. When designing and validating SPR assays for immunogenicity assessment, analysts should consider critical parameters including protein immobilization to ensure immunological reactivity, immobilized protein stability, and surface regeneration conditions.

Protein Immobilization: The first step in the development of immunogenicity assessment assays is to identify the optimum mechanism for immobilization of the target protein. When considering the target density for immobilization, analysts often recommend that a high-density surface be used. The advantage of a high-density surface is that it maximizes the opportunity that anti-therapeutic antibodies will come in contact with an immobilized ligand. A high-density surface also provides excellent assay sensitivity. An important aspect of these assays is that the chemistry chosen for immobilization should provide random orientation rather than a site-directed orientation so that all potential epitopes on the therapeutic protein are available for binding by the anti-therapeutic antibodies. The effectiveness of immobilization is determined by evaluating the ability of positive control antibodies to bind to the immobilized protein. When evaluating the effectiveness of immobilization, analysts should test multiple antibodies with different epitope specificities. When panels of antibodies that cover a range of affinities and bind to different epitopes on the target protein are all capable of binding, this provides confidence that antibodies contained in clinical specimens also will be detected. If any of the positive control antibodies do not demonstrate binding, this suggests that the immobilization is not optimal and should be modified. Although SPR is demonstrably efficient at detecting low-affinity antibodies, analysts should confirm that the immobilization protocol chosen is effective for detection of low- and high-affinity antibodies.

Protein Stability Upon Immobilization: The positive control antibody must be able to bind to the immobilized protein in order for an assay result to be acceptable. This confirmation of binding provides confidence that if antibodies against a protein are present in a sample, they will bind to the immobilized protein on the surface of the sensor. Because SPR relies on re-using the immobilized protein surface for multiple analyses, a regeneration protocol is required to effectively remove any bound material from the immobilized protein. This regeneration procedure is based on the ability to remove bound material without damaging or removing the immobilized protein. Several regeneration protocols can be used, and most often the regeneration solution is an acidic solution, commonly dilute HCl. The immobilized protein must remain intact and functional after repeated regeneration steps.

Because the immobilized protein will be used routinely for multiple analyses involving repeated cycles of serum samples, analysts should verify the stability of the immobilized protein after regeneration cycles. The stability of the immobilized protein can be monitored effectively by tracking the response units after regeneration and also after addition of positive control antibody. If there is a change in baseline or a decrease in the magnitude of binding by the positive control antibody, then the immobilized protein likely is no longer suitable for further analyses. The stability following regeneration should be established during assay development and should be confirmed during assay validation. In order to monitor the performance of the sensor during an assay, analysts should periodically test a positive control sample during an assay run. If the performance of the positive control samples indicates the immobilized protein has been compromised, analysts should re-analyze test samples obtained after the performance of the assay dropped below acceptable limits. Acceptance parameters for immobilization may vary by compound and should be established for each assay.

Availability of Epitopes After Immobilization: Once the protein is immobilized, the availability of multiple epitopes should be confirmed. Ideally this is done by testing for binding of positive control antibodies with different epitope

General Chapters

specificity. One method for testing epitope availability is to use a panel of monoclonal antibodies that are known to recognize different regions of the protein. If the protein has been randomly immobilized, all the different positive control antibodies should be able to bind. The reason for evaluating epitope availability is to prevent false-negative results when serum samples are evaluated. If the immobilization is not random, it would be possible to consistently immobilize the protein via a specific epitope, thus making that epitope unavailable for binding by an antibody. Another possibility is that chemical modification of the protein to facilitate immobilization altered the protein's conformation.

Surface Regeneration and Subsequent Protein Stability: Using the previous guidelines, analysts should monitor the surface for signs of accumulation and degradation of the immobilized ligand and discontinue use when necessary. For example, when the binding capacity of a positive control antibody (diluted in test serum) drops below 80% of initial capacity the surface should not be used.

Assay Cut-point Determination: When performing assays to determine if a serum sample contains antibodies against a protein, analysts sometimes observe a background level of binding. That background binding can vary depending on the nature of the immobilized protein and also the patient population being tested. In order to determine if a test sample contains antibodies, analysts compare binding to control samples that do not contain antibodies against the protein. A cut-point is established, and when a sample contains antibodies the binding is greater than that cut-point. Analysts determine the assay cut-point by analyzing a series of serum samples that do not contain antibodies against the immobilized protein and then performing statistical analysis to determine the level of binding consistent with a sample that does not contain antibodies. The cut-point should be established using the same conditions that will be used for sample analysis. Although different approaches are used for determining a cut-point, a common approach is to establish the mean from the binding of 50–100 serum samples from healthy volunteers and set the cut-point at 95% (equivalent to the mean plus 1.645 times the standard deviation for a normal distribution). Analysts should remove statistical outliers from the calculations because their inclusion can cause a high bias and raise the cut-point. This higher cut-point will result in identification of fewer samples with antibodies against the immobilized protein. The statistically evaluated cut-point is the response unit value that serum samples must exceed to be considered positive for the presence of antibodies against the therapeutic protein. An important feature of cut-point determination is that it may be different in different patient populations. For example, patients with inflammatory disease, may show a higher level of nonspecific reactivity compared to a normal population. This higher level of nonspecific binding would result in samples being identified as positive when they did not contain any antibodies specific for the protein. When this situation arises, predose serum samples can be used to establish a new patient population–specific mean and assay cut-point.

Analytical Procedure Development and Validation: Once the stability of the immobilized protein is confirmed, a regeneration procedure has been defined, and the cut-point established, the antibody testing method can be developed and validated. The conditions used for analyzing samples should be identical to those used to establish the assay cut-point. An important parameter to consider is the optimal dilution of the serum sample. Increasing the dilution factor reduces nonspecific binding by serum proteins but also reduces overall sensitivity. Most antibody assessment procedures use between 5% and 50% serum. As the percentage of serum that is tested decreases, the percentage of the binding signal that is due to nonspecific interaction also decreases, and subsequently the percentage of the signal mediated by antibodies binding to the immobilized protein increases. Besides dilution, other means to reduce nonspecific interaction include adding surfactants, increasing salt concentration, adding BSA or HSA, or adding soluble sensor surface support material such as carboxymethyl dextran or alginate to the dilution and running buffer.

Other important variables to optimize include flow rate and sample volume. The combination of flow rate and sample volume defines the contact time, the length of time during which a given sample is in contact with the immobilized protein. The longer a sample is in contact with the immobilized protein, the greater the chances for antibody binding. The next important aspect to consider is verification that initial binding is a result of an antibody and not some other serum component. This can be accomplished by adding an anti-human immunoglobulin reagent and monitoring subsequent binding. If the initial binding observed was due to an anti-protein antibody, this reagent will bind to that antibody (the anti-protein antibody remains bound to the immobilized protein). When a therapeutic monoclonal antibody is the immobilized protein, the confirmatory reagent must be screened and verified not to bind directly to the immobilized therapeutic monoclonal antibody. One option here is the immobilization of the Fab' fragment rather than the intact therapeutic monoclonal antibody. The confirmatory reagent must be verified for specificity. Once all of the parameters are optimized, the assay can be validated. Validation parameters include those typically associated with immunogenicity assays (precision, specificity, sensitivity, and robustness) as well as parameters specific to SPR assays (protein immobilization, stability of immobilized surface, and number of regeneration cycles).

Interference by Serum Components: Depending on the immobilized protein, serum components other than antibodies specifically directed against the immobilized protein possibly could bind to the immobilized surface. It is also possible that serum components that block the ability of antibodies to bind to the immobilized protein could be present. Both of these can be evaluated by testing the binding of serum samples from the target subject population that are known not to contain antibodies against the immobilized protein and then monitoring to determine if any binding does occur. If nonspecific binding is identified, steps can be taken to reduce or eliminate it. These steps can include pretreatment of samples to remove the nonspecific reactant, addition of surfactant, or alteration of salt concentration in sample buffers to reduce nonspecific binding.

General Chapters

Analysts should verify that serum samples do not contain agents that are capable of inhibiting antibody binding to the immobilized protein (these could include soluble forms of the immobilized protein or soluble receptors that could bind to the immobilized protein and block binding of the antibodies to the immobilized protein). Analysts can add the positive control antibody to target serum samples and can evaluate binding. If binding is inhibited by the target serum samples compared with binding to normal human serum samples, steps can be taken to remove the inhibiting agent. Failure to identify target serum interference can result in either false-positive or false-negative results.

Implementation of Multiplex Assays: When a therapeutic protein is a second-generation product that has been modified from an original therapeutic protein (e.g., via pegylation or increased glycosylation), the presence of antibodies against both the original and the second-generation product should be evaluated simultaneously. This can be accomplished by immobilizing each protein on separate channels in the microfluidic device and allowing serum samples to bind in series or in parallel to both immobilized proteins. The rationale for testing for binding to both the original and the modified therapeutic protein is that antibodies generated against the modified protein could have binding specificity to the original protein as well. As part of the characterization of the immune response, analysts must understand the specificity of antibodies for both first- and second-generation products. When possible, binding to an endogenous counterpart might also be tested by immobilizing the endogenous protein on a separate flow cell or channel.

Characterization of Anti-Therapeutic Protein Antibodies: Once antibodies against a therapeutic protein have been captured by binding to the immobilized therapeutic protein, those antibodies can be characterized. The important features of anti-therapeutic antibodies that can be studied include the relative binding affinity, the amount of antibodies present in the serum sample, the isotype(s) of antibodies present in the sample, and binding specificity.

By monitoring the rate at which the response units decrease after the conclusion of sample addition to the sensor, analysts can determine the relative affinity of the antibodies. A high rate of dissociation is characteristic of a low-affinity antibody, and a slow rate of dissociation suggests the presence of high-affinity antibodies. It is useful to compare the dissociation rates with both the positive control antibody (typically a high-affinity antibody preparation) as well as a panel of monoclonal antibodies of known binding affinities.

The relative active concentration of antibodies present in a sample can be estimated by comparing the binding signal with the signal produced from a dilution series of the positive control. Analysts can generate a standard curve from the standard and can compare the active concentration of antibodies in the sample with that standard curve. Because the positive control does not exactly mimic the mixture of antibodies contained in the sample—in fact, the positive control is often obtained from hyperimmunized animals such as rabbits—the concentration value obtained is relative to the standard. This value only approximates the actual concentration of human antibodies. Because the same positive control can be used throughout clinical development, analysts can compare the amount of antibodies obtained from different subjects using this strategy. Because the instrument's signal is proportional to the mass that is binding to the sensor, this type of analysis provides value. Analysts should consider that IgM antibodies have five times the mass of IgG antibodies. Another approach for determining the concentration of antibodies is described in the concentration analysis section of this chapter (see Application 2 below).

The isotype of captured antibodies can be readily determined by monitoring binding associated with sequential addition of isotyping reagents. For example, if IgM antibodies are present and have bound to the immobilized protein, the addition of an anti-human IgM reagent will produce an additional signal. Isotyping reagents can be found with specificity towards IgM, IgG, IgE, IgA, IgG1, IgG2, IgG3, and IgG4. Because of steric hindrance, analysts may be required to repeat isotyping analyses in different sequences to be certain the presence of previously bound isotyping reagents has not hindered subsequent analyses. For example, assume a sample contains both IgG1 and IgG4 antibodies against a protein and both species have bound to the immobilized protein. Because the anti-IgG1 isotyping reagent has bound to the IgG1 antibodies, the isotyping reagent bound to them may prevent subsequent additions of an anti IgG4 reagent from binding to the IgG4, and the presence of the IgG4 would be undiscovered. Analysts will conclude that only IgG1 antibodies are present, but if the order of isotyping reagent addition were reversed the IgG4 antibodies would be discovered. This example underscores the importance of careful interpretation of isotyping results. This is a problem for subsequent analysis only if there is observed binding by a previous cycle of isotype reagent addition. The specificity of isotyping reagents should be confirmed before use. Analysts should, for example, verify that an anti-human IgG reagent binds only to human IgG and does not cross-react with human IgM.

The region of the therapeutic protein recognized by the antibodies can sometimes be determined by immobilizing versions of the protein that have been truncated, have point mutations, or contain only a fragment of the protein. If the antibodies fail to bind to the changed version of the protein, it suggests that the epitope toward which the antibody is directed was influenced by the change. It should be kept in mind that point mutations and truncations not only influence the primary sequence of a protein, but can also influence the tertiary structure (i.e. folding, conformation) of a protein. Also, a subject is likely to generate a population of antibodies with different specificities for a variety of epitopes. Despite this concern, the strategy just described can prove useful for identifying the region on the protein where the antibodies are binding.

Application 2—Concentration Analysis: SPR can be used to determine the concentration of biologics in defined buffer systems, e.g., eluates from purification columns, formulation buffers, and complex mixtures such as serum, fermentation broths, crude cell extracts, and cell suspensions. The concentration of an analyte is measured by its binding to the specific ligand or other molecules that can interact with any portion of the analyte. The analyte concentration is determined

General Chapters

on a surface where the analyte-specific ligand or an analyte-specific capture reagent is immobilized. The binding rate or the mass of analyte bound is determined, and the analyte concentration is calculated using either a standard curve obtained from a concentration series of a purified and well characterized reference material or by a calibration-free analysis that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration.

Immobilization of Ligand: To determine concentration the ligand is immobilized covalently or non-covalently on the surface. Analysts select an appropriate coupling mechanism and chemistry to ensure the ligand's functional integrity. In order to provide conditions that favor partial or full independence of kinetic parameters, a high surface density of the ligand is desired.

A high-density surface allows the analyte to bind to the ligand under conditions that limit mass transport. The interaction between the analyte and the ligand can be described by the following two-step process:

$$A \xrightarrow{k_m} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$

where A is the concentration of analyte in the sample, $A_{surface}$ is the concentration of analyte at the sensor surface, k_m is the mass transport coefficient, B is the immobilized ligand, AB is the analyte–ligand complex, and k_a and k_d are the rate constants for the reaction between A and B. The mass transport coefficient k_m depends on the flow rate, the dimensions of the flow cell, and the diffusion coefficient of the analyte. The analyte first must be transported from the bulk to the sensor surface to react with the immobilized ligand molecules. If this mass transport of the analyte is much faster than the association step between ligand and analyte, the overall observed binding will be driven by the kinetic rate constants of A and B, a prerequisite for accurately determining the kinetic parameters. If the mass transport of analyte is much slower than the association step, the binding will be limited by the mass transport process, and kinetic parameters for the specific interaction between A and B will be difficult to obtain. However, these conditions are desired for determining the active concentration of an analyte. A high density of ligand on the sensor surface and slower flow rates favor limited mass transport. Between these extremes, the overall binding is determined by contributions from both mass transport and interaction kinetics. It may not be possible to achieve limited conditions of mass transport for concentration analyses of interactions with relatively slow association rate constants (e.g., $k_a = 10^4$ $M^{-1}s^{-1}$).

The ligand and the analyte reference material should be of sufficient purity with special attention to the presence of aggregated material. Aggregates of the analyte can interfere with the regeneration of the ligand surface because they can bind with multiple binding sites.

The reference material must be comparable (e.g., molecular weight and kinetic parameters) to the test samples. Under certain conditions the active concentration in unknown samples can be determined using a calibration-free procedure that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. These two methodologies are described separately below.

Concentration Determination with a Reference Standard Curve: In typical concentration-determination assays the analyte concentration is calculated from a standard curve that is obtained with a reference material injected at select concentrations. Three different approaches can be used to measure concentration with a reference standard calibration curve:

Direct Binding Assay: Determine the quantity of analyte bound after an arbitrarily fixed sample injection time. A sandwich method can be performed as an extension of the single-step direct binding approach in order to increase assay sensitivity.

Binding Rate Determination: Determine the initial binding rate for a sample rather than the amount bound. Under conditions of mass-transport limited binding, the binding rate is directly proportional to analyte concentration, and is independent of binding kinetics. This allows one to measure the concentration of related molecules that might have different binding characteristics.

Inhibition or Competition Assays: When the mechanism of action for an analyte is binding to a soluble ligand and thereby disrupting a ligand–receptor interaction, an inhibition assay can be used. In an inhibition assay, a receptor is attached to the sensor surface by a covalent linkage. The interaction between the analyte and the soluble ligand is indirectly measured by mixing a fixed concentration of ligand with varying concentrations of the analyte and injecting the ligand–analyte mixture across the immobilized receptor surface. Competitive methods in solution can also be used for large molecules and particles such as viruses, as well as for small analytes that give low direct responses. In parallel systems the assay can be designed so that the standard samples and the unknown sample are injected in parallel. This method can be useful for ligands that are difficult to regenerate.

Analysts can plot the signal (amount bound or rate of binding) of the reference material standards against concentrations and then can generate a standard curve using an appropriate mathematical model such as a linear or a logistic four-parameter curve fit. Samples can be injected at one or more dilutions. Fewer dilutions can be employed if a linear relationship between sample and reference standard has been demonstrated. Concentrations of unknown samples are either obtained by back-calculation from the standard curve or, if they are analyzed at the same target concentrations as those of the reference standard curve, by comparison of curve-fit parameters.

Parameters that can influence assay performance and results include but are not limited to flow rate, ligand density on the surface, sample purity, sample matrix, and reproducibility of surface regeneration. These parameters must be eval-

uated during assay qualification or validation. Interference with binding of analyte to the immobilized ligand can be minimized by salts, detergents, or sensor-surface support material. A commonly used sensor surface consists of carboxymethylated dextran, so the addition of dextran to the sample dilution buffer can minimize nonspecific interactions. Injections over a negative control surface can also be used to mathematically subtract the nonspecific binding data from the data obtained on the positive surface. A qualified or validated concentration determination SPR assay should include QC samples that can serve as measure to determine the accuracy of the standard curve that has been prepared to analyze samples with unknown analyte concentration. They can be conveniently prepared in larger batches, qualified for use with a Certificate of Analysis for the target concentration, and stored in small aliquots under appropriate storage conditions.

After each analyte injection the ligand surface is regenerated and all bound analyte is removed. This regeneration must be strong enough to remove all bound analyte, but the conditions also must leave the immobilized ligand intact so that injections can be compared to each other.

Concentration Determination Without Calibration: Calibration-free concentration assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. By measuring the initial binding rate analysts can derive the analyte concentration if specific properties of analyte and the analytical environment are known. This approach can be useful when no satisfactory reference standard is available.

To determine the analyte concentration in a sample, analysts use the relationship between initial binding rate and analyte concentration. On a sensor surface with a high immobilization level, the initial binding rate (slope) can be described as a function of the molecular weight, the mass transport coefficient k_m, and the concentration of the analyte. Before a sample is analyzed analysts must determine the mass transport coefficient. It depends on the diffusion coefficient (D), flow rate, and flow cell dimensions and is described by the following formula:

$$k_m = 0.98 \times \sqrt[3]{\frac{D^2 \times f}{0.3 \times h^2 \times w \times l}}$$

where D is the diffusion coefficient, f is the flow rate, and h, w, and l are the flow cell height, width, and length, respectively. Flow rate and flow cell dimensions typically are known for a given instrument, and the diffusion coefficient is determined by the size and shape of the molecule by the use of instrument-specific tools, literature references, or experiments, e.g., by analytical ultracentrifugation or light scattering.

In a typical experimental setup the evaluation requires two flow rates. By using measurements at two widely separated flow rates, analysts can assess the influence of flow rate on binding rate. The robustness of the assay is also improved by fitting the data obtained at two different flow rates, which give correspondingly two different values for k_m (because k_m depends on the flow rate), to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

Calibration-free concentration analysis is suitable only for proteins with MW ≥ 5000 Da. It requires fast analyte–ligand association ($k_a > 5 \times 10^4$ $M^{-1}s^{-1}$) and it cannot handle mixtures of analytes with different diffusion properties. The dynamic range of the method is approximately 0.05–5 µg/mL.

Application 3—Kinetic and Affinity Analysis: Because of its ability to detect binding interactions in real time, SPR provides valuable information about the kinetics of complex formation and dissociation. SPR instruments can be used to determine the association rate constant and dissociation rate constant for a particular binding interaction, and these values can be used subsequently to calculate the dissociation equilibrium constant ($K_D = k_d/k_a$). Obtaining K_D from a ratio of k_a and k_d is useful when the binding interaction does not reach equilibrium in a timeframe that is suitable for an SPR binding experiment. For binding interactions that reach equilibrium (rate of complex formation equals the rate of complex decay) in min (vs. h), K_D can be determined directly from a steady-state binding response. The length of time required to reach equilibrium is influenced by the dissociation rate, so quickly dissociating complexes (e.g. $k_d = 10^{-2}$ s^{-1}) will reach equilibrium faster than those that dissociate slowly (e.g., $k_d = 10^{-5}$ s^{-1}). Software programs capable of simulating 1:1 binding kinetics are useful for predicting the length of time required to reach equilibrium. The typical working range for affinity measurements with commercially available SPR instruments is from 10^{-12} M (pM) to 10^{-4} M (µM).

Proper experimental design is required to accurately measure k_a, k_d, and K_D. Several questions must be considered when designing kinetic analysis or steady-state affinity experiments, including:

- Which binding partner should be immobilized?
- How will the analyst immobilize one of the binding partners?
- What type of reference surface should be used?
- How much binding partner should be immobilized?
- Does the binding partner maintain activity after immobilization?
- Is binding to the immobilized binding partner specific?
- What regeneration conditions, if necessary, should be used?

When selecting which binding partner to immobilize for most protein–protein interactions, analysts must consider several factors: (1) the purity and availability of the proteins, (2) the presence of a tag or functional group to aid in immobilization, (3) maintaining biological activity, and (4) the binding valency (e.g., monovalent vs. multivalent binding).

General Chapters

A reference surface is required for all detailed kinetic and affinity analysis experiments. If direct immobilization is used, then a reference surface is created using the same immobilization protocol, omitting the protein during the coupling step. Alternatively, a mutant form of the protein with a modified binding site can be used. The reference surface for high-affinity capture typically consists of either the capture molecule and no binding partner, or uses an unrelated molecule for a mock capture surface. For the specific case of antibody–antigen interactions, an unrelated monoclonal antibody often serves as the capture reagent on the reference surface.

After deciding on the immobilization approach, analysts must decide how much binding partner to immobilize. For kinetic analysis, the primary consideration is to minimize the surface density to avoid mass-transport–limited binding of the analyte molecule to the immobilized binding partner. Analysts also must consider the immobilization level when conducting steady-state affinity analysis because high immobilization levels can cause steric hindrance or can induce secondary effects such as nonspecific binding or aggregation.

Before performing a kinetic experiment or steady-state affinity analysis, analysts must assess the activity of the surface by injecting the analyte molecule at a single concentration. The concentration should be high enough that the equilibrium binding response provides a close approximation of the experimental maximum response (R_{max}). This condition is typically met when the target molecule concentration is at least 10-fold higher than the K_D of the binding interaction. For a protein–protein interaction having a K_D value of 100 pM this means that the target molecule should be injected at a concentration of at least 1 nM. Using the R_{max} equation (*Equations 1* and *2*), analysts can calculate the theoretical R_{max} based on the amount of binding partner immobilized or captured. If the experimental R_{max} exceeds the theoretical R_{max}, then the analyte molecule is larger than expected or the analyte exists in a higher-order structure than expected (e.g., following aggregation or as a multimer). If the experimental R_{max} is significantly lower (<50%) than the theoretical R_{max}, this suggests that the immobilization procedure has compromised the binding site, and an alternative immobilization procedure should be investigated. An advantage of using high-affinity capture instead of direct coupling is that the surface activity typically remains close to 100%, provided that the specific activity of the immobilized binding partner is 100% before capture.

Injecting the target molecule across the reference surface also provides a quick assessment of the amount of nonspecific binding that exists on the sensor surface. Nonspecific binding that is electrostatic in nature can be eliminated or reduced by addition of salt (e.g., 0.5–1.0 M NaCl) to the sample diluent buffer and the running buffer or by using sensor surfaces with a low charge density. Nonspecific binding that arises from hydrophobic interactions can be minimized or eliminated by the addition of detergents such as 0.05% Polysorbate 20 or 10 mM CHAPS to the sample diluent buffer and the running buffer. Before using buffer additives, analysts should test whether the specific binding interaction or binding activity is affected. Nonspecific binding to a capture molecule can be resolved by switching to a different capture molecule. Reducing surface density also may eliminate nonspecific binding.

Many protein–protein interactions dissociate slowly ($k_d = 10^{-3}$ to 10^{-6} s^{-1}), with complex half-lives ($t_{1/2}$) of more than 2 h. A regeneration step is important for these types of binding interactions.

In some protein–protein interactions, surface regeneration may not be possible because of a high-affinity binding interaction between the molecules. If this situation occurs, analysts can consider a titration kinetic experiment or the use of instruments that are capable of performing parallel analyte injections. In a kinetic titration, increasing concentrations of target molecule are injected consecutively across the immobilized surface, and the resulting data are analyzed using a titration kinetics model. In parallel instruments the analyte concentration series is injected in one step, thus eliminating the need for regeneration.

Surface regeneration typically is not performed for steady-state affinity experiments because the binding interactions in this type of experiment have k_d values in the range of 10^{-2} to 0.5 s^{-1}, and therefore have $t_{1/2}$ values of <2 min. The bound analyte dissociates from the surface as buffer flows over the surface, and regeneration is not required.

Analysts should have an accurate analyte concentration for kinetic analysis because the calculation of k_a depends on the analyte concentration. Typically an absorbance reading at a wavelength of 280 nm (A_{280}) is used for this purpose, but analysts should remember that the A_{280} value reflects the total bulk protein in solution and does not reflect the actual concentration of protein that is capable of binding (i.e., the active concentration).

Sample diluent injections should be included in replicate for kinetics and steady-state affinity experiments so that nonspecific responses due to instrumentation or sample diluent can be removed during the data-evaluation process.

The recommended analyte concentration range is 10-fold below and above the K_D for the interaction. By keeping the surface density low (R_{max} = 5–50 RU) and extending the association time, analysts should be able to collect data with enough curvature to accurately define k_a and k_d values. When the affinity of the interaction is high (K_D = low nM to pM), higher analyte concentrations may be required to build a kinetic profile with sufficient curvature to complete the kinetic analysis. Increasing the analyte concentration should not be a substitute for changing other experimental design parameters (e.g., surface density and contact time). Although it is desirable to use analyte concentrations that approach R_{max}, unusually high concentrations may induce aggregation of the analyte in solution or nonspecific binding to the surface.

Within an analyte concentration series, replicate samples should be used to assess surface activity. Additionally, each concentration series should be tested 3–5 times using different surfaces in order to establish confidence intervals for the resulting kinetics and affinity constants.

The amount of data that is collected during the kinetic or steady-state affinity experiment affects the accuracy of the results. For kinetic experiments involving binding interactions with slow k_d values, analysts should collect enough dissociation data so that a measurable dissociation response (vs. instrument noise) is acquired. For example, a binding interaction between a therapeutic monoclonal antibody and its target molecule that has a k_d of 10^{-5} s^{-1} will require the collection of at least 4 h of dissociation data. Rather than collecting this much dissociation data for all analyte concentrations, analysts can use a long dissociation time for the highest concentration of analyte that will be injected, and they can use a short dissociation time (2–5 min) for all other analyte concentrations. With parallel-injection instruments one can collect the long dissociation data for all of the concentrations. For data evaluation analysts should collect both long and short dissociation time data for sample diluent injections. For steady-state affinity experiments, the analyte injection time should be long enough to allow for a steady-state binding response to occur at all analyte concentrations that are tested. Dissociation data do not have to be collected for a steady-state affinity experiment because dissociation data are not used in the evaluation of this type of experiment, but the complete dissociation of analyte is required before beginning the next injection.

Use of SPR in a Regulated Environment

When SPR assays are used for lot release and stability testing, the assay must exist within a controlled setting so that decisions can be made about the use of product within the clinic or marketplace. SPR instrumentation, including software, should be 21 CFR Part 11 compliant and should be amenable to validation. These requirements are important because they help ensure the integrity of both data acquisition and data evaluation.

Besides using SPR instrumentation that meets regulatory requirements, analysts should establish system suitability criteria for an SPR assay. Including these criteria in an SPR assay ensures that the results obtained for the test sample are generated by an assay that is performing within its operating parameters. A discussion of assessing system suitability parameters is not within the scope of this chapter, but the reader is referred to USP general chapters *Design and Analysis of Biological Assays* ⟨111⟩ and *Analysis of Biological Assays* ⟨1034⟩ for more detailed discussions. Some examples of system suitability parameters for an SPR assay can include:

- ligand immobilization density
- parameters from a curve fitting model (e.g. four parameter logistic curve fit)
  - — EC_{50} values for reference standard curve and positive QC curve
  - — Effective asymptotes (response range) for reference standard curve and positive QC curve
  - — R^2 values for reference standard curve and positive QC curve
  - — parallelism between reference standard curve and positive QC curve
- relative bioactivity for positive QC (EC_{50} ratio of reference standard to positive control)
- calculated concentration for positive QC (single-point positive QC measurement)
- binding response for negative QC (nonspecific analyte or diluent)

Multiple lots of ligand, analyte, coupling reagents, and sensor surfaces should be used to establish assay system suitability criteria that reflect normal assay conditions. Assay results for reference standard and QC samples should be tracked over time. Regular trending analyses should be done on the data to show whether the SPR assay remains in control over its required lifecycle. If trending in the data is observed, remedies can be performed proactively, preventing assay failure.

For SPR assays that are used in lot-release testing, it is also important to establish sample acceptance criteria. These criteria are used to accept or reject sample data and can include:

- relative bioactivity for test sample
- coefficient of variation (CV) for sample replicates
- parallelism between reference standard curve and positive QC curve

Another point to consider for SPR assays in regulated environments is the identification of noncritical and critical reagents. Noncritical reagents typically include coupling buffer, regeneration buffer, and continuous flow (running) buffer. Critical reagents typically include the ligand and analyte for direct binding assays, and ligand, analyte, and competitor molecule in inhibition binding assays. Critical reagents should be qualified/requalified on a routine basis to ensure that they are suitable for use in the SPR assay. Best practices for the characterization of critical reagents are not within the scope of this chapter, so the reader is referred to current regulatory documents for such discussion. Analysts should use current regulatory documents such as ICH Guideline Q2(R1) Validation of Analytical Procedures and *USP–NF General Chapter Validation of Compendial Procedures* ⟨1225⟩ when they validate SPR assays in a regulated laboratory.

⟨1106⟩ IMMUNOGENICITY ASSAYS—DESIGN AND VALIDATION OF IMMUNOASSAYS TO DETECT ANTI-DRUG ANTIBODIES

INTRODUCTION AND SCOPE

Anti-drug antibodies (ADA) can be induced when animal or human immune systems recognize a protein drug product as foreign. The administration of biopharmaceuticals can elicit product-specific ADA, and various types of ADA responses can develop in either nonclinical or clinical studies. [NOTE—A list of regulatory documents, white papers, and other relevant references is contained in the *Appendix*.] Although the main focus of this chapter is ADA immunoassay design and validation, the chapter also includes discussion of an overall risk-based immunogenicity assay testing strategy that includes preclinical and clinical studies.

ADA assay results are directly influenced by assay design, assay reagents, how the assay is run, what samples are run in the assay (timing of sample collection, etc.), and how assay data are analyzed. In fact, it essentially is impossible to compare the results of studies that use different ADA assays. Guidance, such as this general information chapter, recommending best practices and considerations for ADA assay development helps ensure that the assays produce results that are meaningful for patient safety and product efficacy.

The primary concern with unintended or unwanted immunogenicity of biological products is whether antibodies produced by patients receiving the product lead to some form of clinical response (e.g., an effect on safety or efficacy). The utility and interpretation of preclinical toxicology studies also can be influenced by the presence of ADA.

The pharmacokinetics (PK) or the pharmacologic activity of the drug can be altered by ADA that either enhance or reduce the clearance of the drug, alter bioavailability, or inhibit or exacerbate the pharmacological action of the drug. If an endogenous counterpart of a drug exists, ADA that inhibit the activity of the product also can bind to and cross-react with an endogenous protein counterpart of the product, potentially leading to a deficiency syndrome. Under some circumstances, ADA can form immune complexes that can induce serum sickness-type clinical responses. Moreover, IgE isotype ADA responses can result in anaphylaxis.

Immunogenicity assessments are playing an increasing role in biopharmaceutical development as part of Product Quality Risk Assessments (PQRAs) and the assessment of the criticality of quality attributes (as described in ICH Q8 and Q9). They also play a role in the demonstration of process and product comparability after manufacturing process changes. Often the manufacturing process for a biological therapeutic will be refined during clinical development, and often changes occur after the sponsor obtains marketing authorization. Such changes, however minor, potentially could affect the bioactivity, efficacy, or safety of a biotherapeutic, and immunogenicity is a key consideration. Changes in the levels and types of degradation products (oxidized, deamidated, aggregates, or others), isoforms of the protein, and process-related impurities such as host cell protein and DNA could affect immunogenicity and warrant closer evaluation.

FACTORS THAT AFFECT THE IMMUNOGENIC POTENTIAL OF A THERAPEUTIC PROTEIN

Many factors can influence whether administration of a biological product will induce an immune response in the recipient, including the structure of the protein product itself, product variants, product formulations, the immune status and genetic makeup of the patient, and the dosing route and regimen used in the clinic.

Protein Structure

The primary amino acid structure of the product and its variants can determine if there are immunogenic epitopes that the patient's immune system recognizes as foreign, leading to an immune response. Amino acid sequences that are not found in human proteins and thus could be recognized as foreign by the human immune system (e.g., those derived from a nonhuman cell line or created by protein engineering, e.g., fusion proteins) not surprisingly can induce an immune response in humans. In addition, chemical modification of amino acids (e.g., oxidation or deamidation) may result in a sequence that can stimulate an immune response, although few data to date have confirmed such occurrences following administration of therapeutic proteins. Truncation of the protein could expose amino acid motifs (neoepitopes) not normally exposed in the native protein, stimulating an immune response.

In general, glycosylation does not appear to play a major role in the induction of immune responses to biological products, although nonhuman (e.g., murine) or nonmammalian (e.g., products derived from plants) glycosylation can induce immune responses. An example is a human monoclonal antibody that contained a terminal galactose-α-1,3-galactose because of post-translational modification by its murine production cell line. This antibody was antigenic and caused severe hypersensitivity reactions in presensitized individuals bearing cross-reactive IgE. There was no evidence that this glycan induced primary immune reactions in naïve (i.e., not presensitized) individuals. Such examples are rare. In fact, in many cases complex carbohydrates may prevent or reduce the antigenicity of immunogenic proteins by shielding epitopes from binding antibodies.

Aggregated protein has been shown to induce immunogenicity in animal models. Proposed mechanisms of action include either presentation of high molecular weight, repeating subunits (multimers) directly to B-cells thereby inducing a T-cell–independent response or by inducing a T-cell–dependent response via enhanced antigen presentation. Addition of polyethylene glycol (PEG) molecules (PEGylation) to recombinant therapeutics has been attempted in order to attenuate immunogenicity and antigenicity of recombinant proteins by limiting exposure of epitopes. Although no clear evidence establishes that the immunogenicity of proteins is diminished by PEGylation, some clinical data suggest that PEGylation can limit antibody binding (i.e., antigenicity) to the protein backbone. PEG itself can be immunogenic and anti-PEG antibodies should be monitored, especially in subjects with known PEG hypersensitivity. In fact, a background level of anti-PEG antibodies is present in the general population, leading to the requirement to develop an anti-PEG antibody assay as well as an ADA assay during the clinical development of PEGylated products.

Process-Related Impurities

Process-related impurities such as endotoxins, host cell DNA, or proteins can act as adjuvants and can provoke an immune response by evoking danger signals via activation of immune cellular receptors such as Toll-like receptors. Some postulate that leachables from primary packaging components also can act as immune stimulators or affect the higher-order structure of the protein product and induce an immunogenic response, although firm data are not yet available.

Immune Status of the Patient

The ability of the patient's immune system to recognize and respond to a protein product can dictate the level of immune response. Patients who are taking immunosuppressive drugs such as glucocorticoids, cyclosporine, or methotrexate may have a lower likelihood of immune response to a protein product despite its immunogenic potential. Conversely, autoimmune diseases and inflammation may involve the overactivation of an immune system so that a product's level of immunogenicity may be much greater than one would anticipate. The pharmacologic activity of the protein therapeutic itself should also be taken into account. Some protein therapeutics directed against B-cell antigens can deplete peripheral B-cells. Conversely, other protein therapeutics may have immune-modulatory activities, e.g., altering patterns of T-cell trafficking. These activities may affect an individual patient's or a patient population's ability to mount an immune response to a protein product.

A patient's immune status should also be considered when the patient exhibits specific pre-existing ADA, cross-reacting ADA (for example, in the case of murine- or plant-derived products), or antibodies against production cell line-related impurities that might induce a clinical response.

Genetic Background of the Patient

Molecules that recognize and present protein-derived peptides to the adaptive immune system—the human leukocyte antigen or major histocompatability antigen molecules—show considerable genetic diversity between individuals and between populations in different parts of the world. This diversity is one reason why different patients may have different immune responses to the same product. Consequently, if clinical studies include only a population of limited genetic diversity, then the immunogenicity profile of a protein therapeutic in that population may not reflect its immunogenicity profile in the larger, more diverse population that would be exposed to product after approval.

Dose and Route of Administration

The way a therapeutic protein product is used can influence its potential for immunogenicity. Different routes of administration appear to have different effects on immunogenicity, and subcutaneous injection generally is perceived to be a more immunogenic route of exposure than is intravenous administration. The dosing regimen also can influence immunogenicity. A protein therapeutic that typically is administered one time as a single dose (e.g., a thrombolytic protein) is less likely to induce an immune response than is a protein therapeutic that is used in a multidose regimen because the immune system usually requires a prime followed by a boost to ensure a robust response. Patients may have pre-existing sensitization even without any known exposure to a therapeutic protein product, and they may exhibit adverse clinical responses on first exposure. However, products with long half-lives or those that are particularly immunogenic (e.g., those with multiple T-cell epitopes) have been shown to induce ADA after a single injection. A chronic dosing regimen has a greater chance of inducing an immune response because the immune system receives multiple exposures to the product and this can lead to a strong memory T-cell response.

Another regimen that has caused ADA with clinical sequelae is one whereby a product is given for a short period of time, stopped, and then introduced again only after a long lag period. This has the effect of priming the immune system, and the reintroduction can cause immune-related events such as allergic responses. The use of chronic dosing on a regular basis, although it repeatedly provides the drug product, appears to avoid such hyper-responsiveness by inducing some form of tolerance.

DETERMINATION OF PRECLINICAL AND CLINICAL IMMUNOGENICITY

Preclinical: Relevance and Scope of Preclinical Immunogenicity

The immunogenicity of many biotherapeutics is greater in preclinical studies and has low predictive value for humans because an immune response to human or humanized proteins tends to be greater in animals than in humans because of the perceived foreignness of the protein in animals and the absence of an endogenous biological counterpart. Even though detection of ADA in animals may not be clinically relevant, researchers must assess ADA for the interpretation of the required toxicity data necessary for regulatory submissions (see ICH S6R1 in the *Appendix*).

Generally ADA assessments, PK data, and pharmacodynamic (PD) data aid in the interpretation of the results and validity of animal toxicology studies. In some instances preclinical immunogenicity data also can be used to compare the relative immunogenicity of products before and after manufacturing changes, although only with the grossest of changes in relative immunogenicity does this appear to be meaningful. Preclinical studies, particularly in higher animal species, are typically not statistically powered to draw conclusions on relative immunogenicity but, when they can, it should be recognized that differences in MHC restriction and natural immune tolerance of animals versus humans may not permit translation of such information to effects in humans. Furthermore, it should also be acknowledged that strains of preclinical species are limited in genetic diversity compared to the human population.

ADA may alter exposure to an active drug by blocking the therapeutic agent from binding to the target or by accelerating the clearance of the therapeutic agent from circulation, resulting in reduced exposure. ADA may increase the half life of biologic drugs and with this the exposure if the drug–ADA complex is still bioactive. Typically, samples should be collected for possible ADA analysis from all preclinical safety studies in which animals are exposed to the drug for more than 7 days (see references in the *Appendix* for more information). Because the key consideration for including immunogenicity analysis in nonclinical studies is to demonstrate the exposure to the drug for the duration of the study, this also can be achieved by demonstrating drug-mediated modulation of a PD marker. In addition, an absence of an effect on the serum concentration or PK profile of the drug also can indirectly demonstrate the absence of detectable effects of an ADA on drug exposure. However, in some situations the development of an ADA may not significantly affect the clearance of the drug, but instead the ADA may affect the drug's binding to and activity at the target (e.g., anti-idiotype antibodies). Therefore, lack of an effect on a PD marker and/or on PK also should be considered to ensure activity is not affected by the ADA.

For most nonclinical studies, samples from various phases of the study should be collected, banked, and analyzed with regard to any observed pharmacological or toxicological changes. In fact, most nonclinical toxicology studies do not evaluate the kinetics of ADA development, and samples for the assessment of ADA usually are taken at baseline, end of treatment, and end of recovery periods.

Analyses of samples taken during the dosing phase also can be performed if unusual PK data or toxicological findings are observed at the end of the study. In this case it needs to be taken into consideration that analysis of samples taken during the dosing phase may be complicated by drug interference. Therefore, it is important to take samples at appropriate time-points (e.g., before the next dose) and to have suitable assays with high drug tolerance in place. In some instances when a soluble target may be present in the circulation, understanding of ADA level (titers or relative mass units) can facilitate the interpretation of toxicological findings.

A risk-based approach should be used to determine if further characterization (e.g., demonstrating neutralizing activity) is necessary for preclinical studies. Factors such as the presence of endogenous counterparts, utility of a PD marker, or the PK assay may affect this decision. If data from a neutralizing antibody assay (NAb) are deemed necessary, then this assay could be either a target-binding inhibition immunoassay or cell-based assay, irrespective of the risk level. Immunogenicity evaluation and study data interpretation typically require serial sampling and analysis of serum samples for PK, PD, and ADA. Such repeated and frequent sampling may not be feasible when researchers conduct studies using rodents, particularly mice. In such cases, the study can be designed to allow discrete analyses of toxicity, PK, PD, and ADA endpoints from similarly treated cohorts of mice with sample collection at similar study time points to allow inferential analysis of effects observed across treatment groups.

Clinical: Relevance and Scope of Immunogenicity Assessments

In clinical studies, ADA detection and characterization is important to understand the safety and exposure and efficacy profile of the therapeutic. Typically, the ADA analysis strategy in clinical studies involves a screening assay for binding antibodies then a confirmatory assay, followed by further characterization in NAb. Immunogenicity data from clinical studies generally are analyzed in the context of their relevance to the PK and PD of the therapeutic and on adverse events. For replacement therapies (e.g., enzyme-replacement therapies, blood clotting factors, and erythropoietin), a comprehensive monitoring program should be designed based on ADA detection combined with multiple safety parameters to monitor the potential for serious adverse events.

Researchers should consider the kinetics of the appearance of ADA during clinical studies because this can affect not only ADA detection but also the potential clinical sequelae. Some products induce antibodies rapidly, but other biotherapeutics can take years before an immune response is detected or can be correlated to any clinical sequelae. Investigators also should consider whether an antibody response is transient or persistent. Therefore, understanding the kinetics of ADA appearance is im-

portant. This goal can be achieved by carefully selecting ADA sample collection times and taking care in developing the ADA assay for clinical studies. Samples should be collected during each phase of the study (pretreatment, during treatment, and during any washout phases). The sensitivity and drug tolerance of the ADA assay also must be appropriate for the intended use of the assay. For example, when products have long terminal half-lives (e.g., monoclonal antibodies) scientists should develop ADA assays that are capable of detecting ADA in the presence of product levels that are expected to be present in patient test samples. In addition, during the design of ADA sampling plans, researchers should consider the appropriateness of obtaining samples after a washout period.

The number of patients assessed for ADA in clinical trials and the duration and timing of ADA sample collection are critical factors to understand the incidence and clinical impact of ADA. Other factors may confound ADA analysis, including the nature of the therapeutic itself, the presence of pre-existing cross-reactive antibodies, rheumatoid factors, heterophilic antibodies, soluble targets, or ligands. Samples should be taken to assess the levels of these interfering factors in serum (and other PD markers as applicable) at the same time as ADA samples.

Besides determining the presence of binding antibodies, clinical immunogenicity assessments typically include further characterization of positive samples in titer assays as well as in NAb to determine the potential effect of ADA levels to neutralize the drug's effect or to mediate safety events. In addition, understanding the kinetics of ADA and NAb development by detection of ADA in sequential samples taken throughout the study phases and elucidation of the ADA immunoglobulin class(es) and subclass(es) may aid in better understanding of patient- and treatment-related factors and the mechanisms by which the therapeutic induces ADA development.

RISK-BASED APPROACH TO ASSESSING IMMUNOGENICITY AND ITS CONSEQUENCES

Assessing the Potential Risk of Product-Specific Immunogenicity

The concept of risk is defined in ICH Q9 as the combination of the probability of occurrence of *harm* and the *severity* of that harm. In relation to pharmaceuticals, the protection of the patient by managing the risk to safety should be considered of prime importance and thus risk assessments of product-induced immune responses should focus on the potential severity of clinical consequences from ADA responses rather than the probability of occurrence of ADA responses. A few patients with severe or life-threatening ADA-related side effects are of more concern than many ADA-positive patients who have no clinical consequences. Risk mitigation should also be factored into the overall risk-assessment process (e.g., elimination of clinical impact by co-medication).

Although ADA testing strategies can be based on immunogenicity risk assessment, this may not always be feasible during early drug development when reliable assays may not be available.

ADA-induced safety events can range from mild side effects to life-threatening conditions. The potential severity of the consequences of an ADA response should be considered as early as possible. *Table 1* summarizes some but not all of the risk factors that may influence the severity of clinical consequences from an ADA response.

Table 1. Factors That May Influence the Severity of ADA-Related Clinical Sequelae

	Lower Risk[1]	Medium Risk	Higher Risk
Presence of an identical endogenous counterpart (includes families of proteins that share domains)	No endogenous counterpart (e.g., Botulinum toxin)	Redundant counterpart (e.g., interferons or growth hormones)	Nonredundant counterpart [e.g., erythropoietin or megakaryocyte growth and development factor (MGDF)]
Presence of a structurally related endogenous molecule or domain	No structurally related endogenous molecule or domain	Medium structurally related endogenous molecule or domain exists	Highly structurally related endogenous molecule or domain exists
Patient's disease state	Life-threatening	Moderate-to-severe	Mild
Potential clinical consequences of immunogenicity	No clinically meaningful impact on safety or efficacy	Manageable impact to safety and/or efficacy	Extensive clinical influence on safety or loss (or dramatic increase) of efficacy

[1] Risk is a consequence of both severity and frequency.

A number of factors may contribute to the incidence of an ADA response, and some but not all of these factors are shown in *Table 2*. During an immunogenicity risk assessment the factors shown in *Table 2* should be considered in conjunction with those in *Table 1*. The clinical consequences of immunogenicity are unpredictable, even with the risk assessments outlined above.

An immunogenicity risk assessment is of real value only when all the factors that influence the likelihood and severity of a potential immune response are carefully considered. Risk assessments should be done in a cross-functional manner, including input from clinicians, safety assessment, PK, bioanalytical scientists, as well as process scientists. Consultations with regulators and clinical safety monitoring boards also may be helpful and should be carried out iteratively during the product development process as clinical data are obtained.

ADA-mediated clinical sequelae and ADA incidence rate are separate entities, but the two factors are interrelated because the number of patients with ADA-mediated serious adverse events may rise with a higher ADA incidence rate.

Table 2. Factors That May Influence ADA Incidence

	Lower Incidence	Medium Incidence	Higher Incidence
Circulating level of endogenous counterpart	Abundant	Scarce	None
Patient's immune status	Suppressed	Normal	Activated
Exposure: dosing regimen or frequency	Single dose	Chronic (maintenance) Multiple dosing	Episodic dosing
Route of administration	Intravenous or oral	Subcutaneous, intramuscular, mucosal (non-inhalational)	Intradermal or inhalational
Product Characteristics	Lowest levels (or absence) of product or process-related impurities (e.g., aggregates or denaturation, fragmentation)	Intermediate levels of product- or process-related impurities, and potential epitope content	Higher levels of product- or process-related impurities (e.g., aggregates; denaturation, fragmentation)
	Molecular integrity of active substance maintained		Higher level of antigenic epitopes (derived from murine cell lines, contains novel mutated sequences, etc.)
	No or low content of potential T-cell epitopes		Mechanism of action: immune activation
	Mechanism of action: e.g., immunosuppressant		

Risk-Based Approach to ADA Testing Strategy

The ADA testing strategy should be based on an immunogenicity risk assessment. Appropriately designed, validated, and executed immunogenicity assays and testing schemes provide the data that make risk assessments possible and predict the eventual outcome for patients. The frequency of sampling, neutralizing activity assessments, and qualitative or quasi-quantitative measurements may all depend on perceived risk.

In clinical studies, patient safety is of primary concern, and the extent of ADA characterization necessary depends on the potential risk of ADA-related sequelae. The type of drug should also be taken into account. For example, for a multicomponent fusion protein that contains at least one component with a potentially high risk of adverse events, a domain-mapping method (i.e., reactivity of ADA with individual components) is recommended.

Generally, more extensive ADA testing and characterization should be applied if the risk of clinical adverse effects is high. Samples should be analyzed and characterized based on the timing and incidence of the ADA response as well as the occurrence and severity of clinical side effects. A higher risk of ADA incidence normally does not warrant extensive characterization of ADA, and usually the risk of clinical consequences drives the bioanalytical strategy. Nevertheless, some investigations may be driven by the need to understand the cause of a high ADA incidence (e.g., the reactivity of ADA with aggregated versus nonaggregated drug).

The following step-wise, risk-based testing strategy can be refined depending on the product's level of risk and during the design of the clinical studies to ensure that the maximum amount of data can be gained, including correlations to PK, PD, etc.

STEP 1

Develop ADA methods that are fit for purpose and are consistent with current industry best practices and regulatory guidance. Incorporate baseline and postdose ADA testing into the clinical study design, together with concurrent testing for PK plus any relevant PD, safety, or efficacy markers that will facilitate interpretation of ADA data. The analysis of results from ADA testing should be built into study analysis plans.

STEP 2

Test all pre- and post-dosing samples for ADA. Two important tests that should be carried out in all cases are the ADA screening assay and the drug inhibition or immunoglobulin depletion confirmation assay. Report as negative any ADA results below the assay cut-point and with drug levels below the interference levels, as well as those that test negative in the confirmatory assay. Test methods that are capable of sensitive ADA detection despite the presence of trough levels of drug are desirable. In their absence, samples containing drugs above the interference levels should be reported as inconclusive with a statement of possible drug interference. In such cases, ADA analysis could be performed later following a drug washout period to reach a conclusive result (refer to the section *Relative Sensitivity* of this chapter for further information). For the confirmed positive samples, ADA levels should be estimated, preferably by titration, but they can be reported in terms of relative mass units. Certain mass-based technology platforms may necessitate the use of relative mass units.

STEP 3

Samples deemed positive in *Step 2* should be tested for neutralizing ability and potentially other characteristics, depending on the risk assessment. In high-risk situations, NAb activity should be measured, typically using a cell-based assay. Depending on the drug's mechanism of action, sometimes a ligand-binding NAb assay format can be used if it is adequately proven to specifically detect NAb. Concurrently generated PK/PD/safety or biomarker data should be used to help interpret the clinical relevance of neutralizing antibody activity. In addition, determination of ADA isotypes and affinity may be helpful in the overall assessment of the immune response. Allergic reactions associated with drug administration may necessitate measurement of drug-specific IgE, although detection may depend on the sampling scheme and method design.

DESIGN OF IMMUNOASSAY-BASED TEST METHODS

Immunoassay methods for ADA detection generally are complex and require a broad understanding of multiple technical challenges. Screening assays that serve as the key first step in the immunogenicity testing scheme are designed to have a certain false positive (rather than false negative) rate in order to maximize the sensitivity for detecting ADA. In addition, using a risk-based approach, it is more appropriate to have 5% false positives rather than false negatives during this initial screening phase. Typically, positive screening samples are confirmed to contain drug-specific ADA in confirmation assays before the determination of the level of ADA (titers) or any additional characterization.

Screening Assays

In their simplest form, screening ADA assays immobilize the therapeutic protein on a microtiter plate or onto beads to capture ADA (solid phase) or co-incubate a labeled therapeutic protein at a predetermined concentration with the sample containing ADA (solution assay). The bound polyclonal ADA is then detected using a labeled secondary reagent or labeled drug. Limiting the number of wash steps or reducing wash fluid dispensing rates may increase the detection of antibodies with fast off-rates on assay platforms that use wash steps. Generally, screening assays are designed to detect classes of antibodies that may be most relevant to the product's route of administration, e.g., IgA for mucosal routes of administration. Although the most common ADA raised against protein therapeutics are of the IgM and IgG isotypes, other isotypes of ADA including IgE and IgA may require detection based on the clinical response and the route of product administration. In addition, depending on how rapidly ADA responses develop and the half-life of the therapeutic, it may be feasible to detect the development of ADA initially of the IgM isotype that later affinity matures to an IgG isotype following repeat administration of product.

Because screening assays serve as the first step in the immunogenicity testing program, these assays generally are configured to have moderate throughput and often are automated. The various technology platforms used to develop screening immunogenicity assays have inherent strengths and weaknesses as outlined in *Table 3*. Development of a bioanalytical strategy to use a certain technology platform for assay development should take into consideration the nature of the product (e.g., a therapeutic protein or a monoclonal antibody), potential sources of interference in the assay (e.g., therapeutic concentration anticipated in patient samples, soluble target based on co-medications, and biology), and disease-specific interfering or cross-reacting factors (e.g., rheumatoid factor).

Table 3. Advantages and Disadvantages of Various Assay Types Currently Used to Assess ADA

Assay Type	Advantages	Disadvantages
Direct/Indirect ELISA	High throughput	Potential for high background
	Readily available	May not be specific
	Easy to automate	Utility depends on capability to detect different Ig subtypes
	Inexpensive	Drug tolerance lower for solid-phase ELISAs
	Readouts can increase sensitivity (e.g., electrochemiluminescence)	Excessive washes can decrease detection of low-affinity ADA
	Drug tolerance higher for solution-phase ELISAs	
Bridging Format	Low background	Difficult to confirm presence of IgM
	Highly specific	Need to label product
	Readily available	Reduced ability to detect low-affinity antibodies and IgG_4 because of single-arm binding between bound ligand and detector
	Easy to automate	
	Inexpensive	
	Format can be used across species and detects all isotypes	
	Can be used across detection platforms (colorimetric, electrochemiluminescence, etc.)	

Table 3. Advantages and Disadvantages of Various Assay Types Currently Used to Assess ADA *(Continued)*

Assay Type	Advantages	Disadvantages
Surface Plasmon Resonance/Biolayer Interferometry Assays	Flexibility to characterize immune response (concentration and relative affinity)	Expensive technology
	Higher drug tolerance and can detect low-affinity antibodies	Limited suppliers
		Moderate throughput
Radioimmunoprecipitation Assays	Inexpensive	Radioactive waste
	Highly sensitive	Need to frequently requalify radiolabeled reagents because of short half-life
	Better for high-affinity antibodies	Utility depends on capability to precipitate all relevant antibody present
		Can be less useful for low-affinity antibodies

In addition, analysts should consider whether the method does not result in an underestimation of ADA-positive samples because the ADA binding epitopes on the capture reagent are blocked with a tag or by coating on plates or beads. Another point for consideration in the design and development of an ADA assay is the adaptability of the test for both nonclinical and clinical matrices. Although most assay formats can transfer readily from nonclinical to clinical use, there are exceptions. Further, clinical ADA assays should be qualified or validated for use with samples collected from a similar patient population. Here again, although most ADA screening assays may not show unique matrix interferences between one disease matrix and another, there may be exceptions. Immunoassays used for ADA detection generally are quasi-quantitative methods because standardized, species-specific (especially human) polyclonal ADA calibrators generally are not available. The positive controls, typically developed in-house as hyperimmune serum in animals or by phage display serve as surrogates for the drug-induced ADA in treated patients.

As depicted in *Table 3*, some of the more common assay formats currently in use for development of screening assays include plate-based or bead-based enzyme-linked immunosorbent assays (ELISA; see also USP general chapter *Immunological Test Methods—Enzyme Linked Immunosorbent Assays (ELISA)* ⟨1103⟩) with colorimetric, fluorometric, or luminescent read-outs, plate-based or solution-phase electrochemiluminescent (ECL) or ELISA assays, surface plasmon resonance assays (SPR; see also USP general chapter *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩), or biolayer interferometry assays, and radioimmunoprecipitation assays (RIPA). In order to differentiate positive from negative responses, assay cut-points are statistically determined using samples collected from the target population. The assay cut-point also helps to determine the assay sensitivity. An incorrectly established cut-point may result in false negatives or too many false positive responses that should be ruled out as drug-specific responses in the confirmation assay. Assay performance typically is optimized during development by evaluation of the following parameters: sensitivity (lowest amount of detectable antibody in a sample demonstrated using surrogate controls); specificity (likely detection of a true positive rather than a nonspecific interaction); precision (reproducibility of results from multiple analyses); interference (interfering substances in sample matrix, including the administered drug, that affects assay sensitivity); and stability and robustness (likelihood of optimal assay performance over time). After optimizing these parameters, analysts typically validate the method for its intended use. If the initial assay cannot meet the performance goals (e.g., because of poor sensitivity or high backgrounds), then analysts should either improve and validate the first assay format again or develop and validate more than one assay format.

Confirmatory Assays

Samples that are positive in the screening assay usually are confirmed in a second assay that includes adding a certain fold excess of the therapeutic. This is intended to demonstrate that a positive signal seen in the ADA screening assay is caused by the presence of drug-specific antibodies. Because the cut-point for the screening assay is set to result in the detection of approximately 5% false positives, the confirmatory assay is used to rule out the false positive samples from further analysis. Multiple options are available for performing confirmatory assays. Usually a soluble drug is added to the sample and should compete with the plate-bound immobilized drug for binding to sample ADA. A specific interaction to a soluble drug results in a decrease in the assay signal. As with the screening assay, the cut-point for the confirmation assay is established statistically. Verification of the presence of drug-specific antibody can also be performed using an orthogonal method on a different assay platform that may have different nonspecific binding profiles. Analysts should take care while adopting this approach to ensure an adequately sensitive assay in order to avoid a false negative reaction. Finally, the specificity of ADA to a drug can also be confirmed by depleting all immunoglobulin from a sample (e.g., using a Protein A or G column) followed by reanalysis of the depleted sample. In the latter approach, the depleted sample scores negative in the assay if an antibody caused the original signal. Validation of the confirmatory assays helps ensure that the results from the confirmatory assay are appropriately interpreted.

Characterization Assays

Following screening and confirmation, the relative level of ADA in a positive sample is assessed by titration. The most common approach is to serially dilute the sample and report the reciprocal of the highest dilution factor at which the sample tests

positive, or the titer of the sample. The higher the sample dilution (and therefore the ADA titer value), the higher is the concentration of circulating ADA in that sample. This approach has been used historically to report serological data from vaccine studies. Another less frequently used approach is to express the amount of ADA in the sample in mass units relative to a surrogate standard. Although this approach has the advantage of relating the amount of antibody in the sample to assay sensitivity, analysts should recognize that the calibrator may not be representative of the polyclonal ADA responses under measurement.

Traditional approaches to dilutional linearity testing do not apply to ADA assays. However, when analysts express ADA data in terms of titer values, they also should demonstrate that the positive control(s) displays reasonable (relative) linearity of dilution.

In addition to performing titration, analysts routinely characterize positive ADA samples in neutralization assays to determine the in vitro effect of ADA that might reflect the in vivo biological or pharmacological activity of the therapeutic product. Additionally, the isotype(s) of the ADAs also can be analyzed. Isotype(s) identification is sometimes performed in a multiplexed manner. Using one fluorescent multiplex platform, analysts mix each sample with multiple secondary reagents that are specific to different immunoglobulin isotypes and are labeled with unique fluorochrome labels.

An SPR-based platform also can be used for this purpose (also see *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩). The isotype of an ADA can be determined by observing binding of isotype-specific reagents (such as an anti-human IgG_1 Ab) to the ADA that has been captured by immobilized drug. Analysts should take care when identifying and validating the isotype-specific reagents because unexpected cross-reactivity often is observed. Isotyping can help understand the maturation of the immune response. For example, an ADA response that is comprised solely of IgM antibodies is an immature immune response without T-cell involvement and may or may not progress further. In contrast, an immune response comprised of IgG_1 and IgG_4 antibodies represents a more mature response that has already engaged more components of the immune system. ADA titration and characterization assays are validated routinely using many of the same parameters as screening and confirmation assays to ensure consistent assay performance.

VALIDATION OF IMMUNOASSAYS

Method validation is a process of demonstrating, by the use of specific laboratory investigations, that the performance characteristics of an analytical method are suitable for its intended use (see also USP general chapter *Validation of Compendial Procedures* ⟨1225⟩). The level of method validation depends on the stage of product development and the risks associated with the product. A partial validation involving assessments of method sensitivity, specificity, and precision requirements with less emphasis on robustness, reproducibility, and stability may be adequate for the earlier stages of clinical development (Phase 1–Phase 2 studies), whereas fully validated methods are required for pivotal and postmarketing studies.

Validation of an assay before use of the method for sample bioanalysis is called *pre-study validation*, and amendments to this process may be made between studies. This process maps out the performance characteristics of the assay and should demonstrate that the method is suitable for its intended purpose when it is subsequently applied to study samples. In contrast, *in-study validation* refers to the monitoring of assay performance during study-phase applications of the assay in order to ensure that the assay remains valid and that the resulting bioanalytical data are reliable.

Reliable performance of the assay also depends on all the elements spanning bioanalytical testing and data manipulation, such as assay reagents, analysts, equipment, and computer programs. In essence, the assay is a system comprising several elements other than assay steps and reagents alone. Pre-study validation therefore establishes system suitability (establishment of criteria for control samples that are used to accept or reject runs and imprecision limits for individual samples), and then in-study validation continues to verify it. Critical changes to methods often require additional validation (partial or full), sometimes leading to the revision of the system suitability criteria.

Minimum Required Dilution

During assay development the minimum required dilution (MRD) can be defined as the dilution level of the ADA negative sample that results in the highest signal-to-variability ratio (or Z' factor).

The ability to dilute such samples also should be assessed to ensure that the chosen MRD is adequately distal to any prozone (hook) effects that may have been observed. Although they are rare, some unusual prozone effects may require the test method to include more than one dilution of a test sample to minimize false negative data.

The MRD should be evaluated during the assay development/design phase, i.e., before analysts initiate the validation experiments, so analysts do not need to repeat the evaluation during validation. It can be established using 10 individual drug-naïve ADA negative samples, each tested in 2-fold serial dilutions (e.g., a range of 1:5 to 1:80). The MRD is sometimes defined as the dilution level that results in the highest signal-to-background ratio when typically the background is the dilution matrix. However, this definition ignores the variability in the signal. Therefore it is preferable to define the signal-to-background value and include variability.

One way of doing this is to use a Z' factor that includes both the intensity of the assay readout and its variability at different dilutions (see the *Appendix* for more information). The Z' factor for each dilution level is obtained from the formula [(mean (S) − 3 SD (S)] − [mean (B) + 3 SD (B)]/[mean (S) − mean (B)], where S is the assay signal of the diluted sample and B is the background signal. Thus, the MRD is the dilution that results in a desired value for the Z' factor and signal-to-background ra-

tio. This metric is widely used for high-throughput screening assays to ensure adequate confidence in the ability to differentiate between truly active versus inactive compounds. An inappropriately large MRD can compromise the sensitivity of an assay.

Pre-Study Validation

Pre-study validation establishes the following:
1. Assay cut-points
2. System suitability criteria
3. Relative sensitivity
4. Specificity
5. Selectivity/interference
6. Precision
7. Robustness
8. Reproducibility
9. Stability

Assay Cut-Points

Because of the quasi-quantitative nature of ADA detection methods, the use of a decision threshold or cut-point becomes necessary to discriminate between ADA-positive and -negative samples. Because the screening assay and specificity confirmation assay produce different types of results, separate cut-points are necessary. In some instances, a different cut-point also may be necessary for evaluating titers of confirmed positive samples from the titration assay. Some key points in the evaluation process are summarized briefly below (more information is available in the *Appendix*).

SAMPLES FOR CUT-POINT EVALUATION

Samples from an appropriate population should be used for the development of assay cut-points. In some cases it may not be practical or feasible to obtain matrix samples from a population that has a target disease before initiating pre-study validation experiments. Consequently, samples from healthy drug-naïve subjects are used commonly to establish the initial cut-points. This approach is preferred for a conventional Phase 1 study with normal volunteers. When the clinical program progresses beyond Phase 1 and samples from the target disease population become available, it is more appropriate to re-evaluate cut-point data for the target population. If the distribution of assay responses with respect to both the mean and variability are comparable between the target population and the normal volunteers, then the same cut-point can be used. If not, target disease-specific cut-points are more appropriate, and fixed or floating cut-points computed from the data obtained from the baseline samples from a clinical trial can be considered.

SCREENING CUT-POINTS

The screening assay cut-point is a signal in the screening assay that identifies a sample that is likely to contain ADA (termed a *screen positive* or *potential positive* sample) versus an ADA-negative sample. A screening assay cut-point is established during pre-study validation based on a systematic and statistically rigorous analysis of assay responses from a panel of individual samples that are considered to be representative of a drug-naïve target patient population.

To determine the screening method cut-points for clinical assays, analysts should use samples from at least 50 drug-naïve individuals for a robust evaluation. If additional indications are targeted, analysts should test at least 20 drug-naïve individuals per indication. If the variability is significantly different from the original indication, then additional drug-naïve individuals may need to be tested. If not, then the original cut-point can be applied to the additional indication. For nonclinical assays a total of at least 25 drug-naïve individuals should suffice. To ensure this cut-point is robust, at least two analysts should perform this experiment over three days in at least two different plate layouts. A balanced experimental design and plate layouts will help avoid potential confounding between analysts, subject samples, run dates, etc. For clinical ADA assays, if multiple disease-state populations are being tested they should be distributed evenly across the plates to ensure they are properly balanced across plates and runs. Statistical outliers of the sample results should be examined and eliminated, e.g., using outlier box plots defined in terms of quartiles and interquartile range. In addition, confirmed reactive samples (e.g., via immunodepletion) can be excluded as well.

Three types of screening cut-points can be calculated for application during the study phase—fixed, floating, and dynamic—and one of these should be appropriately chosen for study phase bioanalysis.

Fixed cut-point—A fixed cut-point is a cut-point that is determined in pre-study validation and subsequently is used for the in-study phase. The fixed cut-point is used for analyses of test samples until there is a need to revalidate or change the cut-point (e.g., because of a critical change in the assay, assay transfer to another laboratory, upgraded instruments, etc.). The cut-point value can be fixed within a given study, for a target population, or across studies and multiple target populations. In order to use this approach, one should statistically demonstrate similar means and variances across runs during pre-study vali-

dation. A fixed cut-point can be determined based on the mean + 1.645 SD (standard deviation), which represents the 95th percentile of the population under normal distribution (and therefore is expected to identify approximately 5% of the samples as false positives). The standard deviation should include different sources of variation such as the intra-run, inter-run, inter-analyst, and inter-subject variability. If the data are not normally distributed, appropriate transformations (typically log transformations) can be used. If transformation doesn't help, usually it is acceptable to determine the nonparametric 95th percentile. However, in preclinical trials it may be considered adequate to use a cut-point at the 99th or 99.9th percentile because immunogenicity of a protein normally results in high antibody titers. Alternatively, even if the validation data suggest similar means and variances across runs, to account for possible deviations between assay runs during the in-study bioanalysis phase, it would be safer to apply a floating cut-point.

Floating cut-point—A floating cut-point is a cut-point calculated by applying an additive or multiplicative normalization factor, determined from the pre-study validation data, to the biological background obtained during the in-study phase (see Appendix G of Shanker et al., 2008, in the *Appendix* for details). The biological background may be represented by the negative control (pool of matrix from subjects that are negative for ADA), the assay diluent, or the predose subject sample (subject-specific cut-point). The method for determining floating cut-point uses the variation estimate from the pre-study validation that includes different sources of variation such as the intra-run, inter-run, inter-analyst, and inter-subject variability. Such a cut-point is recommended when the means of drug-naïve samples are not similar but the variances are similar across runs. When a negative control is used for normalization, analysts also should ensure that the negative control results represent the drug-naïve matrix sample results of the target population. This is accomplished by demonstrating that the signal of the negative control trends directionally with the signal of the individual samples. Alternatively, the use of assay diluent for normalization or pretreatment subject ("baseline") sample results may be more appropriate. However, a pre- versus post-dose ratio might be a better solution.

Dynamic cut-point—A dynamic cut-point is a cut-point that changes between the plates in a run, between runs in a study, or between studies, and it does not apply the variation estimates from pre-study validation. The latter characteristic differentiates it from a floating cut-point. This approach is necessary only where means and variances significantly differ between runs. Because this approach entails testing of several individual drug-naïve samples for the evaluation of a run-specific cut-point, it consumes a large portion of the plates from each in-study run and therefore is not practically feasible, especially when analysts use 96-well plates instead of 384-well plates. Differences in variability between assay runs sometimes can be resolved by further optimization of some key steps in the assay protocol or by resolving some analytical issues. In some cases, the differences in variability can be attributed to different analysts or instruments, and use of an analyst-specific or instrument-specific floating cut-point may resolve this issue. If further optimization does not resolve the situation or if the causes are not clear, another practical alternative may be to pool the variability across all runs and use this pooled variance for floating cut-point evaluation during the in-study phase.

SPECIFICITY CONFIRMATION METHOD CUT-POINT

Because of the conservative approach of incorporating a 5% false-positive rate into the computation of the screening cut-point, the elimination of false-positive samples via confirmation of drug-specific binding is an important component of ADA bioanalysis. It is also important to understand the level, if any, of the drug itself within the sample.

The amount of change in assay signal that differentiates drug-specific binding from nonspecific binding is referred to as the *specificity cut-point*. The specificity cut-point should be determined by an objective approach in the context of assay variability near the low positive range of the assay. To determine the specificity cut-point, drug-naïve negative samples from at least 25 individuals should be evaluated (however, more are commonly used when available), with and without drug preincubation. Ideally these samples should be the same as those tested during the screening cut-point evaluation, and the unspiked and spiked counterparts of the individual subject samples should be tested together in the same plates.

The mean percent change from the unspiked sample (inhibition) and SD are calculated. The mean inhibition plus 3.09 SD (if a 0.1% false positive rate is desired) represents the specificity cut-point. As in determination of the screening cut-point, specifically reactive samples after preincubation with drug (i.e., those that contain pre-existing antibodies) and statistical outliers should be eliminated in order to make the specificity cut-point more conservative. The analytical process outlined above for the screening cut-point applies for the evaluation of the specificity cut-point as well.

Alternative approaches such as the use of *mock low positive* samples in which the individual drug-naïve samples are spiked with a low concentration of a positive control sometimes is considered for this cut-point evaluation. However this method is subjective and is not recommended because it depends on the concentration of the positive control and the unique affinity/avidity of the positive control that may or may not represent true positive patient samples. Additional sources of information regarding the relative statistical merits of these approaches and methods for verifying the assumptions are listed in the *Appendix*.

TITRATION METHOD CUT-POINT

The titration method cut-point is a test result value below which further serial dilution of an ADA-positive sample produces negative assay results. Typically, the screening assay cut-point is used as the titration cut-point, but the validation of a separate titration method cut-point can become necessary when the signal from the assay diluent or matrix causes higher results than

General Chapters

the screening assay cut-point (because of a blocking effect of serum) or if samples at a dilution higher than the MRD do not generate consistently negative results, i.e., when the screening cut-point falls on the lower plateau of the positive-control dilution curve. In such instances, the same data generated during a screening cut-point experiment can be used to define the titration cut-point using a 0.1% false positive rate threshold criterion (i.e., Mean + 3.09 SD). During bioanalysis, confirmed positive patient samples that fall between the screening cut-point and titration cut-point can be assigned a titer value equal to that of the MRD.

CROSS-REACTIVITY METHOD CUT-POINT

If applicable, ADA-positive samples that are confirmed to be specific to the drug can be further characterized for cross-reactivity to other related antigens. Like the specificity confirmation assay, a cross-reactivity test method may require a preincubation step with and without the related antigen. Cross-reactivity to the antigen is confirmed when the percent inhibition of signal in the presence of the antigen is greater than or equal to the cross-reactivity method cut-point. The methods for determining the cross-reactivity method cut-point are similar to those for the specificity method cut-point, although it also may be acceptable to apply the drug specificity confirmation cut-point.

Defining System Suitability

ASSAY CONTROLS

ADA-positive controls can comprise polyclonal or monoclonal anti-idiotypic antibodies. They should be affinity purified and quantitated to enable assay validation. Each run (or plate) should include at least a low level of positive control (*low positive control*) and a negative control, but the inclusion of a higher level control (*high positive control*) also can be useful in monitoring method performance. Tracking all of these controls over time can help ensure that the method is performing suitably. A low positive control helps ensure that the assay remains as sensitive during study phase bioanalysis as during the pre-study validation.

On the one hand, the low positive control should produce a response that can be seen reproducibly above the cut-point, but sometimes it may result in a signal that is below the cut-point (thereby failing or invalidating the assay). On the other hand, choosing an unreasonably high concentration for a low positive control may produce an assay signal that is substantially above the cut-point, which is inappropriate. To provide objectivity to the selection of a low positive control concentration, it is useful to think in terms of assay rejection rates, i.e., the percentage of assays (plates) that fail because the low positive control produces a result below the cut-point. As an example and in order to understand if the low positive control is sufficiently low, a 1% rejection rate may be a reasonable target for a low positive control. This is calculated as mean + $t_{0.01,df}$ × SD, where mean and SD are determined using the data from the sensitivity experiment or related assay development data, and $t_{0.01,df}$ is the critical value determined from the *t*-distribution corresponding to a 1% false positive rate and *df* is the degrees of freedom that depends on the number of samples and runs used in the calculation. This theoretically implies that about 99% of the data from the low positive controls will be at or above the cut-point.

An optional high positive control can be useful for methods that are prone to hook effects, tracking assay performance, reagent qualifications, and troubleshooting. The concentration of the high positive control should be chosen from the upper end of the linear range of the dilution curve, usually just below the upper plateau of the curve.

SYSTEM SUITABILITY CRITERIA

System suitability criteria using assay controls help ensure that an analytical procedure remains valid for use. Acceptance ranges (system suitability criteria) for quality controls should be established by statistical evaluation of the experimental data acquired during assay validation.

When the floating cut-point approach is deemed necessary and is used for the screening cut-point evaluation, the system suitability criteria or limits can be defined for the ratio of the low positive control to the negative control and for the ratio of the high positive control to the low positive control or a negative control instead of defining limits separately for each positive control. It is also useful to apply acceptance criteria for intra-assay precision (variability of signals of replicates in an assay) for the in-study phase. Although data from assays that fail acceptance criteria during the in-study phase should be rejected, setting criteria for passing or failing assays in pre-study validation experiments should be avoided because these potentially can lead to the exclusion of some validation data, resulting in an inaccurate estimate of analytical error. All assays during pre-study validation should be included, and the only exceptions should be those rejected for an assignable cause (e.g., technical error).

Relative Sensitivity

No ADA-positive control can be expected to represent the spectrum of humoral immune responses observed in individuals treated with study compounds. The sensitivity of ADA assays is highly dependent on the nature of the positive control reagent(s) so that high-affinity positive controls often produce better sensitivity values than lower affinity positive controls in the

same assay. Analysts should consider this when they choose controls, as well as when they estimate assay sensitivity. Moreover, because the drug itself can interfere with ADA detection, the sensitivity of ADA detection becomes progressively worse in the presence of increasing concentrations of drug within the sample. Despite these caveats, the determination of assay sensitivity is valuable when analysts choose an optimal ADA detection method or platform, a low positive control for validation, or assess the suitability of an assay. The assessment of assay sensitivity in the presence of an interfering drug (drug tolerance) is critical for understanding the suitability of the method for detecting ADA in dosed patients. ADA assay sensitivity should be defined not as a single value, but as a set of at least two values: (1) the concentration of positive-control ADA detected within undiluted matrix in the absence of any drug and (2) the concentration of positive-control ADA detected within undiluted matrix in the presence of drug levels expected at the time points when samples for ADA analysis are taken. Assays should, in general, demonstrate a sensitivity of at least 500 ng/mL for methods applied to clinical studies (or 1000 ng/mL for nonclinical studies) to show suitability for intended purpose—that is, for the detection of clinically meaningful ADA, although assay sensitivity should be justified on a case-by-case basis. It is not useful to express sensitivity in terms of antiserum titers, and thus sensitivity should be assessed using monoclonal antibody or affinity-purified polyclonal preparations. Analysts can evaluate sensitivity by means of two assay runs performed by two independent operators (when feasible) for a total of at least three runs.

To assess sensitivity in the absence of a drug, analysts should prepare mock samples with known concentrations of ADA that are serially diluted (usually 2- to 3-fold serial dilutions) in matrix pooled from drug-naïve individuals and evaluated according to the screening method until the assay results of the dilutions in matrix are below the screening assay cut-point. The lowest concentration of ADA that is consistently found (for example, using a 95% upper confidence limit based on the number of runs or operators) above the screening assay cut-point is determined to be the sensitivity of the assay. Alternatively, it can be the lowest concentration of ADA that is found to be above the screening assay cut-point in all runs by all operators or in 19 of 20 runs (see the *Appendix* for more information).

To assess sensitivity in the presence of a drug, two alternative experimental approaches could be considered: (1) titrate the drug into undiluted matrix containing set concentrations of a positive-control ADA (e.g., 250, 500, or 750 ng/mL). Report the highest concentration of the drug at which ADA remains detectable. (2) Alternatively, because immunogenicity samples often are taken at drug trough time points, prior knowledge of the anticipated trough drug concentration range could be used to determine the assay sensitivity in the presence of the expected concentrations of the drug.

Specificity

Specificity refers to the ability of a method to detect ADA that specifically binds the drug molecule, its domains, or components. The assay is developed and optimized based on the ability of the positive-control ADA to specifically bind the drug. During validation, results of the specificity confirmation assay support assay specificity.

Selectivity and Interference

The selectivity of an ADA assay is its ability to identify a positive control in biological matrix samples that may contain potential interfering substances and is an important concern for ADA detection assays. Such matrix effects typically arise from nonspecific binding interactions between a matrix-based factor and the ADA or from specific binding of unknown factors. During validation, analysts assess the selectivity of the ADA assay by looking at the recovery of analyte (represented by a positive control sample) from matrix samples that contain the potential interferent(s). One caveat here is that the selectivity of an ADA assay, as assessed using the positive control, may not reflect the selectivity of the assay when it is used with actual nonclinical or clinical samples.

Interference is the property of a factor (most commonly the drug itself and its target, if soluble) to affect assay results positively or negatively. It should be evaluated using a low positive ADA test sample that is spiked into a sample matrix from drug-naïve patients. Each potential interfering factor should be tested at a physiologically or pharmacologically relevant range of concentrations. The highest concentration of the interfering factor that does not alter the classification of the test sample (e.g., an ADA sample that remains positive relative to the screening assay cut-point) is defined as the tolerance of the assay to that interfering factor. For therapeutics that have a long terminal half-life, the main interferent in an ADA assay is the drug itself. As discussed previously, the drug tolerance of an assay should be interpreted as the sensitivity of the method in the presence of interfering drug.

Other endogenous interferents include oligomeric drug targets, or the target's soluble receptor may interfere with ADA detection. In addition, certain sample pretreatments performed to reduce drug interference can release drug target from drug-target complexes, leading to subsequent interference problems. Hence, analysts should carefully evaluate pretreatment steps such as acid dissociation during assay development to mitigate the risk of inaccurate data.

Precision

Precision is a measure of the variability in a series of measurements for the same material run in a method. The acceptance criteria for the precision of ADA assays should be within the range commonly expected for immunoassays. These criteria also should be appropriate for the assay platform and should be fit for purpose. During assay validation, precision should be deter-

mined in experiments that are run at the level of intended use during the study phase (e.g., number of plates, samples per plate, etc.).

The acceptance criteria for precision should be within the range commonly expected for immunoassays. These criteria also should be appropriate for the platform used, guided by assay development data and experience with the technology platform and assay method. Additional information is found in the *Appendix*.

SCREENING AND CONFIRMATORY METHOD PRECISION

For ADA screening assays with numeric readouts (as opposed to categorical yes/no readouts), assay precision can be determined using data from at least six independent assay runs of the assay positive controls (low positive and high positive controls). Typically, estimates of intra-assay precision (interreplicate variability, also called intra-assay repeatability) and interassay precision (also called interassay repeatability, or intermediate, total, or overall precision) are reported as percent coefficient of variation (%CV).

Intra-assay precision (repeatability) is the variability of assay results when the same material is tested multiple times within the same run. Interassay precision (also termed intermediate or total precision) is the variability of assay results when the same sample is tested in separate runs, over separate days, and by multiple operators (or only one operator if the study phase bioanalysis is intended to be performed by only one operator). These are expressed as %CV of ADA signals. Data from the replicates of negative and positive controls from each of all the runs tested during the pre-study validation phase are pooled and analyzed within the framework of random-effects ANOVA, resulting in estimates of intra-assay %CV and interassay %CV. Analysts should consider positional effects by varying sample position on microtiter plates because these effects (e.g., edge effects) can influence the assay precision. One should use the same number of test and control sample replicates during validation as are used in the assay during routine use.

Similarly, intra- and inter-assay precision estimates for the confirmatory assay can be derived using the percent inhibition data of the spiked versus unspiked low positive control samples from multiple assay runs (at least six) in the pre-study validation.

General Chapters

TITRATION ASSAY PRECISION

In order to determine the precision of a titer, two or more analysts should assay serial two-fold dilutions of five or more mock high positive control samples in at least six runs. Mock high positive control samples can be obtained by spiking individual negative sera from the target population with a high positive sample. The titer then is determined by interpolation of each of the dilution curves, and the overall mean and SD are calculated. Then intra- and inter-assay precision (%CV) can be determined.

A recommended but more rigorous approach is to use these data to define a minimum significant ratio (MSR): MSR = $10^{t \cdot \mathrm{sqrt}(2) \cdot \mathrm{SD}}$, where SD is the overall standard deviation (intra-run plus the interrun variation) of the titers in common (base 10) log scale, and t is the threshold from Student's t-distribution with $n - 1$ degrees of freedom (n = number of runs). The calculated MSR reflects the smallest fold-change in the titer values that can be considered as statistically significant ($P < 0.05$)—i.e., if MSR = 5, then titers that are different by more than five-fold can be considered significant. In addition to serving as an indicator of the level of variability in the titers of the positive control, this MSR evaluation also can be an approximate criterion for comparing samples with confirmed pre-existing antibodies in baseline versus posttreatment samples in order to assess treatment-induced immunogenicity. The MSR applies only if the titer is interpolated and does not apply to endpoint titers.

Robustness

Robustness is an indication of the reliability of an assay. It is assessed by the capacity of the assay to remain unaffected by small but deliberate variations in method performance that would be expected under relevant, real-life changes in standard laboratory situations. The choice of robustness variables to test during validation should be based on the knowledge of the assay and its associated risks. Some common variables are microtiter plate lots, incubation times, temperature, and reagent lot and concentrations. Study samples or positive control samples can be used to test assay robustness. The use of acceptance criteria for system suitability controls during robustness validation (computed from the assay development and optimization data or validated system suitability control acceptance criteria) is recommended. Continuous monitoring of an assay during validation and beyond with strict records of key assay parameters (e.g., incubation times, pipetted volumes of critical reagents, operators, etc.) may help identify some of the robustness factor interactions if sufficient data are accumulated.

Reproducibility

Assay reproducibility according to USP general chapter *Validation of Compendial Procedures* 〈1225〉 and ICH Q2(R1) *Validation of Analytical Procedures: Text and Methodology* is the reliability of a method when performed in two or more laboratories. In the context of method transfers and interlaboratory method validity demonstrations, assay reproducibility is the same as a cross-validation.

Reproducibility is applicable only if an assay will be run by two or more independent laboratories during in-study phase bioanalysis. Reproducibility is an assessment of the transferability of an assay, i.e., the validity of testing samples in two or more laboratories and the comparability of data produced by them. Reproducibility assessments do not consider routine changes in an assay such as interequipment or interanalyst imprecision. Such contributors to variability (often referred to as intermediate precision factors) are part of the reproducibility variability.

Study phase acceptance criteria for system suitability controls are established in the originator laboratory (see below) during the original assay validation process. The performance of these controls can be compared across multiple laboratories. When only a single laboratory performs the ADA assay, however, reproducibility need not be validated until the method will be transferred to another laboratory.

Stability

It is useful to understand the optimal storage conditions for assay samples, controls, materials, and reagents, and they should be investigated as part of assay optimization before validation. Later, during assay validation, stability studies should evaluate assay performance following intended storage conditions. Ideally, stability testing conditions should mimic the expected sample, material, and reagent handling conditions, storage temperature(s), and varying lengths of storage time.

MATERIAL AND REAGENT STABILITY

ADA assays are stability indicating with respect to the applicable materials and reagents, and thus separate tests for reagent stability usually are not required for assay validation. During study phase bioanalysis, assay materials and reagents are presumed to be stable if the system suitability controls meet validated acceptance criteria. However, analysts should validate the stability of plates that have been prepared in advance (e.g., coated with capture antibody and blocked) and stored.

SAMPLE HANDLING AND STABILITY

ADA samples typically are collected in a serum or plasma matrix. For samples stored at or below −20°C, the stability of ADA are universally accepted, so this sample storage condition may not require validation. It is generally accepted that an ADA sample in serum or plasma will be stable after three freeze–thaw cycles and up to 2 years when stored at −70°C.

Documentation of Pre-Study Validation

Typically three types of assay-specific documents are created during pre-study validation: an assay validation plan or protocol, an assay method description, and an assay validation report.

An assay validation plan or protocol is recommended before analysts initiate pre-study validation experiments. This document should state the intended purpose of the method, a detailed description of the immunoassay and reagents or materials, a summary of the performance characteristics that will be validated, and a priori acceptance criteria for precision, robustness, stability, and, when appropriate, reproducibility. Some experimental detail and data-handling procedures should be presented in the validation plan because these details provide a clear guidance to the validation analysts and ensure better control over the resulting data.

A method description typically is established after pre-study validation but before the study. This provides a detailed description of the reagents, controls, and equipment needed to run the assay, together with a step-by-step operating procedure and information about processes for data reduction and interpretation. The point at which such a description becomes a Standard Operating Procedure (SOP) is specific to each manufacturer's quality system.

When validation is completed, manufacturers generally conduct a technical peer review of validation data, followed by a validation data audit. An assay validation report is created after the validation work is completed. This documents all of the study validation data, together with information about the methods and batches of reagents that were used. An audited report is approved by management and then is archived.

In-Study Validation

In-study validation (monitoring the maintenance of system suitability) and revalidation are critical components of any bioanalytical method. Hence, the validation of a method actually does not end until the method is retired from analytical use.

For in-study performance of quantitative bioanalytical methods, acceptance criteria for precision and accuracy generally are required. Because accuracy is *not* applicable for ADA methods, monitoring the performance of quality control samples reassures analysts that the assay system is suitable for its intended use, i.e., that the assay remains valid and is performing as well as it did during pre-study validation.

The use of a low positive control ensures the assay remains sensitive. Generally during study sample analysis the intra-assay (interreplicate) precision of the results of positive controls, as well as test samples (with assay signal at or higher than the

screening cut-point), are controlled using system suitability acceptance criteria to ensure that meaningful data are consistently obtained. Results below the cut-point, however, may not be required to meet CV limit criteria.

LIFE CYCLE MANAGEMENT

Management of the performance of immunogenicity assays from initial clinical development through subsequent product life cycle requires a comprehensive understanding of the strengths, weaknesses, and capabilities of the method format, as well as of the critical assay reagents and assay performance characteristics. In addition, a well-defined plan for critical reagent production, characterization and qualification, qualification of suppliers of critical reagents, and characterization and qualification parameters for reagents produced in-house (aggregate level and labeling efficiency) help manage the risk of maintaining the assay and transferring the method to other laboratories.

When there are changes in critical method components, equipment, or the population that is studied with a particular ADA assay, an assay revalidation may be required. The revalidation may cover some or all validation characteristics (i.e., it may be a partial or whole assay revalidation). Use of lots or batches of assay critical reagents that are different from those used in prestudy validation do not require assay revalidation, but they must be supported by appropriate experimental qualification data for the new reagent to ensure maintenance of system suitability.

Another critical aspect of life cycle management is the development of a strategy to bridge clinical data between an existing and an improved assay format. Such changes typically occur in a product's life cycle because of postmarketing commitments or other needs. To facilitate comparison and cross-validation of the existing method to the revised versions, analysts should retain sufficient aliquots of the original lots of critical assay reagents. In addition, archiving of analyte-spiked samples as well as blinded patient samples is useful to bridge between reagent lots and methods in order to minimize drift in assay performance. Analysts should develop a written plan outlining the sort of changes to the existing assay or critical reagents that will warrant an assay qualification versus a cross-validation or full validation. A quality management document should include details such as the number of assays that must be performed, the number of analysts that will be used, required training for analysts, acceptance criteria to demonstrate equivalence between existing and revised methods, data analysis, and reporting method. This information demonstrates the robustness and consistency of the assay following changes. Quality controls that ensure assay equivalence include %CV, tolerance limits, EC_{50} values of slope, titer level, and signal-to-noise ratio. One approach commonly used to demonstrate equivalence of two immunogenicity methods is the demonstration of ≥90% concordance in archived sample results between the existing and revised methods.

Analysts should use archived samples with a range of positive values as well as an appropriate number of negatives to verify that a new assay segregates samples into positive and negative categories in the same manner as an existing one.

Another important consideration for life cycle management of critical assay reagents is the monitoring of long-term reagent stability under different storage conditions. A detailed stability testing plan includes storage temperatures (4°C, −20°C, and −70°C), aliquot volume, freeze–thaw cycles, and acceptable performance characteristics for assay qualification, and results should be documented. In this context, it may be prudent to archive patient samples to demonstrate the long-term stability of the polyclonal ADA response in actual patient samples.

APPENDICES

Appendix 1: Nonclinical Immunogenicity Testing

Ponce R, Abad L, Amaravadi L, et al. Immunogenicity of biologically derived therapeutics: assessment and interpretation of nonclinical safety studies. *Reg Toxicol Pharmacol.* 2009; 54:164–182.

ICH. S6(R1) Preclinical safety evaluation of biotechnology-derived pharmaceuticals. Geneva, Switzerland: ICH; 2011.

Appendix 2: Quality Attributes and Immunogenicity Risk Assessments

CMC Biotech Working Group. A-Mab: a case study in bioprocess development. 2009. http://www.casss.org/associations/9165/files/A-Mab_Case_Study_Version_2-1.pdf. Accessed 01 December 2011.

Shankar G, Pendley C, Stein KE. A risk-based bioanalytical approach for the assessment of antibody immune responses against biological drugs. *Nat Biotechnol.* 2007; 25:555–561.

ICH. Q8(R2) Pharmaceutical development. 2009. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf. Accessed 14 December 2011.

ICH. Q9 Quality risk management. 2005. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf. Accessed 14 December 2011.

Appendix 3: Design and Validation of Immunogenicity Assays

Mire-Sluis AR, Barrett YC, Koren E, et al. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods.* 2004; 289:1–16.

Koren E, Quarmby V, Taniguchi G, et al. Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods.* 2008; 333:1–9.
Shankar G, Devanarayan V, Amaravadi L, et al., Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal.* 2008; 48:1267–1281.
Büttel IC, Chamberlain P, Chowers Y, et al. Taking immunogenicity assessment of therapeutic proteins to the next level. *Biologicals.* 2011; 39(2):100–109.

Appendix 4: Statistical Methods

Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomolecular Screening.* 1999; 4:67–73.
Devanarayan V, Tovey MG. Cut-points and performance characteristics for anti-drug antibody assays. In: *Detection and Quantification of Antibodies to Biopharmaceuticals: Practical and Applied Considerations.* Tovey MG, ed. Hoboken, NJ: John Wiley & Sons; 2011.

Appendix 5: Regulatory Guidances for Clinical Immunogenicity Studies

FDA. Draft guidance for industry: assay development for immunogenicity testing of therapeutic proteins. 2009. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf. Accessed 01 December 2011.
EMEA. Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use. 2012. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf. Accessed 30 July 2012.
EMEA. Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. 2015. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/10/WC500194507.pdf. Accessed 15 October 2015.

〈1106.1〉 IMMUNOGENICITY ASSAYS—DESIGN AND VALIDATION OF ASSAYS TO DETECT ANTI-DRUG NEUTRALIZING ANTIBODY

INTRODUCTION AND SCOPE

Administration of natural source or recombinant biologic medicines may elicit some degree of immune response leading to development of anti-drug antibodies (ADAs) in treated subjects. Neutralizing antibodies (NAbs) are a subset of ADAs that affect the biological activity of the biologic drug product. For the purposes of this chapter, NAbs are defined by their ability to neutralize the biological activity of a therapeutic in an in vitro system. This chapter does not address antibodies that may impact drug clearance. [NOTE—Two helpful references on this topic can be found in the *Appendix*.] Further clinical studies would be needed to assess changes in biologic activity on therapeutic outcomes.

NAbs can alter the biological activity of the therapeutic molecule by binding to one or more epitopes that lie within its active site(s). In addition, NAbs can interfere with active sites through steric hindrance (i.e., binding to areas of the protein that are near the active site), or by allosteric interactions (i.e., binding to a site on the drug and inducing a change in conformation that can interfere with the drug's activity). Therefore, it is important to monitor the immunogenicity of biological therapeutics throughout the drug product development cycle by using sensitive and reliable methods that not only determine the presence or absence of ADAs but also characterize whether they have neutralizing capability. The objective of this general information chapter is to provide practical recommendations on best practices that may be used for risk-based design and validation of anti-drug NAb assays.

As described in *Immunogenicity Assays—Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* 〈1106〉, immunoassays (or ligand binding assays) are typically used to screen for the presence of ADAs. Several assay formats can be used to determine whether a detected ADA is also a NAb. The first assay format, defined here as a functional NAb assay (thus having an actual biological readout), is most commonly used and can take the form of either a cell-based functional assay or non-cell-based functional assay (e.g., a biochemical assay for an enzyme therapeutic). Another major group of assay formats is based on NAb-mediated inhibition of ligand binding between therapeutics and their targets. These assays can take the form of either cell-based binding assays (e.g., where a cell-surface target exists) or non-cell-based binding assays (e.g., where a purified soluble receptor or drug target is utilized).

The decision to use a cell-based assay versus a non-cell-based assay is dependent on factors that include the drug's mechanism of action (MoA) and immunogenicity risk assessment, as well as the availability of relevant and sensitive NAb assays (*Figure 1*). Another important consideration in selecting the assay format is the degree of risk to patient safety that NAb forma-

tion would pose; thus, for therapeutics where antibodies pose a high risk to patients, the assay format should be sufficiently sensitive for detecting clinically relevant NAbs. The speed at which results are required can have an influence on the type of assay selected if the cell-based assay requires extended time to execute. Some practical considerations for making this decision are presented in the section *Risk-Based Approach to Assessing Neutralizing Antibodies and Their Consequences.*

Detection of drug-neutralizing activity in such in vitro assays facilitates improved understanding of any observed clinical effects resulting from altered pharmacological activity. A thorough investigation of the presence of NAbs and their characterization in relation to clinical parameters such as pharmacokinetics (PK), pharmacodynamics (PD), efficacy, and safety is therefore strongly recommended. NAbs would have to be produced at a concentration that is biologically relevant such that the effect of the antibodies could be detected in an in vitro assay, and these antibodies could bind with sufficient affinity to remain tightly bound to the therapeutic. In contrast, a low-affinity-binding antibody that recognizes an active region of a therapeutic protein might not remain bound to the therapeutic, and thus would not readily mediate a biological effect. However, one cannot rule out the possibility of low-affinity antibodies having an effect in vivo, and thus NAb assays should be designed to detect the widest range of antibody affinities that is practical (e.g., limit the number of wash steps).

Although the principles described in this chapter are generally applicable to most commonly used cell-based and non-cell-based NAb assays, modified approaches to assay design and validation may be required for certain products (e.g., enzymes), clinical uses (e.g., different dosing regimens or routes of administration), or patient populations. [NOTE—A list of helpful regulatory guidances and white papers is contained in the *Appendix*.]

FACTORS THAT INFLUENCE THE DEVELOPMENT OF NEUTRALIZING ANTIBODIES

Although it is not entirely clear why biological therapeutics induce NAbs only in some individuals, or why certain therapeutics induce more NAbs than others, research has shown that certain factors are typically associated with the generation of NAbs. The initial low-affinity response to a therapeutic protein (primarily composed of IgM antibodies) is rarely able to effectively neutralize the biological effect of the therapeutic protein. However, antibody response to a therapeutic protein can mature, usually upon repeated exposure. As the immune response matures, more epitopes of the therapeutic protein may be recognized by ADAs leading to an increased possibility of NAbs, as epitopes within the active region of the therapeutic protein are now recognized.

Ultimately, those factors that trigger development of a robust immune response require the trigger of T-cell mediated immune responses. Some of these factors include the presence of multiple T-cell epitopes (linear, 7–9 amino acid sequence recognized in the context of the major histocompatability complex class II or MHC II); specific product attributes, such as aggregation, that would encourage uptake into antigen-presenting cells; genetic sequence differences between the therapeutic protein and endogenous counterparts; degree of immunologic tolerance; and the amount, route, and schedule of the administration of the protein therapeutic. There is also a potential for residual host cell proteins to act as adjuvants. Therapeutic proteins that are very close in amino acid sequence to endogenous proteins are less likely to trigger a robust immune response. When the sequence is identical and the endogenous counterpart is fully exposed to the immune system, a high level of tolerance to the therapeutic protein is anticipated. In subjects with autoimmune disorders, this may not be the case, as tolerance to self-proteins has already been compromised.

NAbs are always defined within the context of the assay that identifies them. Thus, it is possible that not all subjects with NAbs are identified in any program. Also, it is important to recognize that if the sensitivity of a NAb assay improves, then the number of subjects with NAbs that are detected may increase. If the incidence of NAb-positive subjects increases, it is important to determine whether the change is related to assay performance or to an alteration in the product quality attributes of the therapeutic protein. NAb specificity is also important. For assays with relatively poor specificity, baseline (pretreatment) samples may show positive responses; however, such a response may not be due to antibodies directed specifically to the therapeutic under study.

DETERMINATION OF PRECLINICAL AND CLINICAL IMMUNOGENICITY

Nonclinical: Relevance and Scope of Preclinical Immunogenicity

Preclinical toxicology studies are generally used to suggest dosimetry and to define safety margins by evaluating idiosyncratic toxicity and specific target-organ toxicity caused by therapeutics. The primary requirement for these studies is to demonstrate that the animals are exposed to the therapeutic at the intended dosage levels throughout the study. As stated in the ICH S6 Guidance, this can be accomplished by analyzing the circulating drug concentration (PK data) in combination with data on PD markers, and/or by using immunogenicity assessments to facilitate interpretation of the exposure-to-toxicity correlation. As discussed in 〈1106〉, animal test systems have significant limitations in their ability to support specific conclusions on the potential for clinical immunogenicity, especially as it relates to MHC restriction, T-dependent B-cell responses, and the potential to neutralize the pharmacological effect of the therapeutic. However, evaluation of immunogenicity in nonclinical studies may be useful when there is substantial homology of the therapeutic to an endogenous counterpart in animals [e.g., erythropoietin (EPO) and thrombopoietin (TPO)]. Severe thrombocytopenia was reported in several animal species after dosing with autologous TPO molecules, due to development of circulating anti-TPO NAbs. In these studies, NAbs directly affected the pharmaco-

logical activity of both the endogenous protein and the exogenously administered therapeutic, making it difficult to interpret the toxicology study findings.

NAbs are usually directly detected and characterized by in vitro assays. However, a decrease in the expression or activity level of a PD marker may be indicative of NAbs. If confirmation that the PD marker is due to the presence of NAb is desired then this can be confirmed with a NAb assay as a surrogate to infer the presence of NAbs. It is important to confirm that the change in the reduced PD marker is not merely due to clearance of drug from the circulation, an effect that can be caused by ADA, rather than due to "true" neutralization. As part of the immunogenicity evaluation, it also may be useful to characterize the immune-dominant epitopes for such high-risk proteins. Nonclinical sample collection and related considerations are discussed in ⟨1106⟩.

Although the measured incidence and titer of NAbs may not be the same in different animal species, induction of NAbs in multiple species may suggest a higher probability of NAb development in humans where significant homology exists within the biologically active site. Immunogenicity studies in transgenic animals expressing the gene encoding the protein of interest may be useful to help predict the relative potential of various forms of drug product to induce NAbs.

Clinical: Relevance and Scope of Immunogenicity Assessments

Evaluation of immunogenicity, including development of NAbs, remains an important part of the safety assessment in clinical drug development.

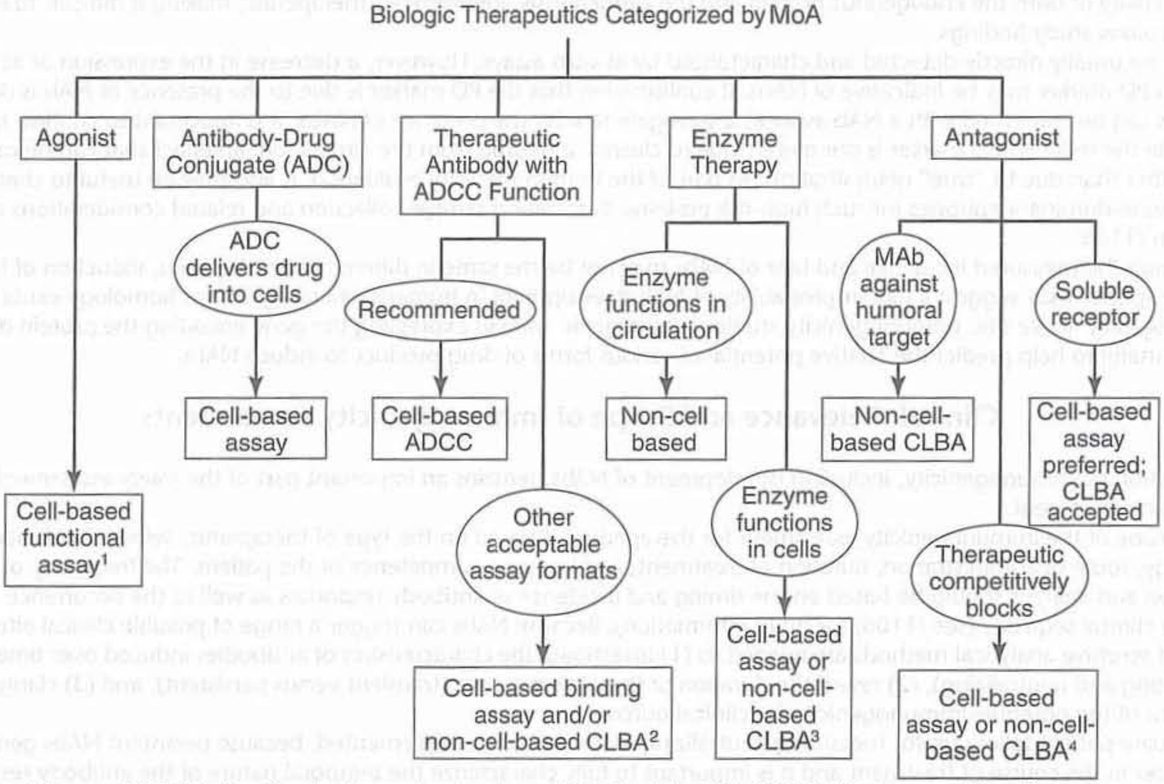
The scope of the immunogenicity assessment for therapeutics is based on the type of therapeutic, selected indication, pharmacology, route of administration, duration of treatment, and immune competence of the patient. The frequency of sample collection and analysis should be based on the timing and incidence of antibody responses as well as the occurrence and severity of clinical sequelae (see ⟨1106⟩ for more information). Because NAbs can trigger a range of possible clinical effects, specific and sensitive analytical methods are needed to (1) investigate the characteristics of antibodies induced over time (non-neutralizing and neutralizing), (2) reveal the duration of the ADA response (transient versus persistent), and (3) clarify the implications of the potential immunogenicity for clinical outcomes.

Adequate patient follow-up for measuring neutralizing ADAs should be implemented, because persistent NAbs generally develop later in the course of treatment and it is important to fully characterize the temporal nature of the antibody response. For example, careful follow-up could reveal a transient response in which antibodies appear, yet then disappear after a period of time such as 2–3 months. In addition to the scheduled, routine repetitive sampling, patients should be evaluated clinically in a symptom-driven manner whenever antibody development is suspected. Pre-approval clinical trials may fail to detect rare immunogenic events due to insufficient patient numbers for adequate statistical evaluation and/or an inadequate duration of drug exposure. Therefore, a postapproval immunogenicity surveillance program may be necessary for any therapeutic protein that carries a risk of generating clinically meaningful NAbs.

RISK-BASED APPROACH TO ASSESSING NEUTRALIZING ANTIBODIES AND THEIR CONSEQUENCES

Chapter ⟨1106⟩ describes in detail the principles of risk-based approaches to detection and characterization of antibodies directed against biologic drug products. The two primary concerns related to NAbs are (1) the therapeutic can lose pharmacological activity, which in turn can affect its efficacy, and (2) NAbs can cross-react with an endogenous, nonredundant protein. In addition, as with all ADAs, NAbs can contribute to other adverse effects such as the formation of immune complexes and potential deposition of these complexes in tissues and the vascular system. *Table 1* in ⟨1106⟩ summarizes important risk factors that may influence the severity of the clinical consequences from an ADA or NAb response. The basic risk assessment criteria outlined in *Table 1* in ⟨1106⟩ provide a useful orientation to this type of risk assessment. The various combinations of factors from the three risk categories (lower, medium, and higher risk), as well as the actual antibody properties, could result in a continuum of intermediate risks. For example, NAbs directed against recombinant human growth hormone can neutralize endogenous growth hormone, which is a vital, nonredundant factor. Yet decades of therapy with a number of different recombinant human growth hormone preparations have shown that attenuation of growth was not observed, even in the presence of the cross-reactive NAbs detected with an in vitro assay. Therefore, the actual risk associated with the recombinant human growth hormone could be viewed as intermediate between the high- and medium-risk categories, despite the existence of the endogenous counterpart.

Potential assay formats are selected based on MoA on a case-by-case basis with proper consultation with regulatory agencies when needed, while the analytical (or immunogenicity assessment) strategy is driven by the risk of immunogenicity for the specific therapeutic. *Figure 1* presents a flow chart with some useful options for NAb assay format selection.



[NOTE—ADCC is "antibody-dependent cell-mediated cytotoxicity;" CLBA is "competitive ligand binding assay;" MAb is "monoclonal antibody."]

[1] A cell-based functional NAb assay is recommended for therapeutic agonists targeting cellular receptor. This includes therapeutics with a nonredundant or redundant endogenous counterpart.

[2] For therapeutics with ADCC function, a cell-based ADCC assay potentially has technical challenges. In addition, ADAs specific to the Fc region of a humanized antibody therapeutic often bind to other nonspecific IgGs in serum matrix and are difficult to detect in a binding ADA assay format. Under this situation, a ligand binding assay using a bridging format or an assay method based on antigen–drug interaction could be a viable platform for the NAb assay.

[3] Non-cell-based assays should be considered, in consultation with a regulatory agency, if the NAb-induced inhibition of lysomal enzyme therapeutics that function at low pH is not feasible under cell-based assay conditions.

[4] For therapeutics that function by binding to more than one receptor, a cell-based assay is recommended.

Figure 1. NAb assay format selection examples.

For agonistic therapeutics that interact directly with cellular receptors, such as growth factors and hormones, development of cell-based NAb assays is appropriate to reflect the MoA of the drug. This category includes therapeutics with a nonredundant endogenous counterpart that may generate clinical sequelae with a high level of risk to patients. For these therapeutics, assays with the capability to determine NAb levels with high specificity and sensitivity are required; the results are generally expressed in titers or equivalents of neutralized drug. These recommendations stem from the concern that NAbs induced by therapeutic proteins can cross-react with and neutralize vital endogenous homologs and cause autoimmune-type deficiency syndromes [e.g., that seen with megakaryocyte growth and development factor (MGDF) and EPO]. Early detection and continued testing for NAbs directed against high-risk molecules are also recommended because NAb data can influence therapeutic decisions.

For antagonistic therapeutics (e.g., anti-IgE or anti-coagulation factors), some regulatory agencies have accepted non-cell-based competitive ligand binding assays for detection of NAbs that are directed against therapeutics with humoral targets. However, for antagonistic therapeutics that interact with a receptor and a coreceptor to exert a pharmaceutical effect on target cells, cell-based assays are generally recommended as this type of MoA cannot be reflected accurately in a non-cell-based ligand binding assay because an ADA that does not block drug–target interactions may interfere with target coreceptor interactions, causing functional neutralization.

In terms of other therapeutics, such as antibody-drug conjugate (ADC), antibody therapeutics with effector functions for clinical efficacy, and enzyme therapeutics that function in target cells, cell-based assays are generally recommended to reflect

the drug's MoA. However, for lysosomal enzyme therapeutics that function at pH 4–5, it may be challenging to generate any NAb controls that can inhibit the enzyme at low pH with sufficient sensitivity. In this situation, the development of a non-cell-based assay (e.g., inhibition of binding between an enzyme drug and its cognate receptor) is warranted in consultation with a regulatory agency. In addition, a non-cell-based enzyme activity assay could be appropriate for enzyme therapeutics, even those that exert their activities within cells. However, results of such an assay will be conclusive only if the conditions required for the intracellular activity of an enzyme drug also allow for the effective binding of a putative NAb to the drug.

Generally, more frequent NAb testing (e.g., monthly or bimonthly) is recommended for high-risk situations. Sampling should be extended post-study for patients who test positive until they test negative in two consecutive tests at least 2 months apart, or based on the half-life of the ADA (e.g., 3–5 half-lives). This may include post-marketing testing. For medium- and lower-risk situations, testing should be conducted on case-by-case basis in consultation with regulatory agencies.

To fully assess the potential risk associated with NAbs, it is also important to understand the exposure–response relationship of a given biotherapeutic, because this relationship could suggest the extent to which NAbs can affect drug activity in vivo. For example, a high dose of drug would be more difficult to neutralize because more NAb would be necessary; thus, there is a lower risk of efficacy loss. In contrast, when a low concentration of drug is sufficient to achieve therapeutic effect, low levels of NAbs could neutralize drug activity, hence increasing the risk level. Similar situations can occur when the concentration of an endogenous drug counterpart is low; even low levels of NAb could neutralize its physiological function and thereby increase the antibody-related risk.

DESIGN OF NAb TEST METHODS

General Considerations

The selection of a NAb assay type is heavily driven by the MoA of the therapeutic, as well as the nature of its target. An example is a monoclonal antibody therapeutic that binds to a receptor on the cell surface, which in turn affects interactions with other cell surface receptors. For this situation, the analyst should use a cell-based NAb assay because it is unlikely that such interactions could be mimicked on a microtiter plate surface. *Figure 1* shows various selections of NAb assays, categorized by their dependence on the MoA of the therapeutic. NAb assay results are generally reported as quasi-quantitative values using a titer, or are qualitative, with just a positive or negative result. Certain technology platforms report quantitative values, such as a surface plasmon resonance (SPR) assay (see ⟨1106⟩ and *Immunological Test Methods—Surface Plasmon Resonance* ⟨1105⟩) or in special cases, neutralization of specific amounts of drug. It could be argued that any numerical value of sample neutralizing capacity is quasi-quantitative when using an antibody preparation as a reference standard, because the affinities and nature of the antibodies within the standard are unlikely to mimic exactly what is contained in a test sample. In addition, comparing sample values between different laboratories that use different standard preparations will likely yield different results for the same sample. Therefore, although a NAb sample value in mass units is helpful for making relative comparisons, all the caveats associated with that value should be considered. A titer value is recommended as the primary readout for NAb assays and is created by testing a sample using serial dilutions. The reciprocal of the test sample dilution that produces a positive result, as defined by the assay cut-point, represents the assay titer. This assay titer should be multiplied by the initial minimum required dilution (MRD; see also ⟨1106⟩), if not accounted for, to report the titer of NAbs present in the neat sample matrix.

Cell-Based Methods for NAb Assessment

As stated previously, cell-based NAb assays can have two general types of readouts: one based on binding events and the other requiring a series of intracellular events that lead to a functional readout. Drug binding to a receptor or surface ligand on the cell can result in its internalization, phosphorylation, or changes in cyclic adenosine monophosphate (cAMP). Functional assays have more complex cellular readouts, such as proliferation, reporter gene expression, or protein synthesis and secretion.

As shown in *Figure 1*, if a cell-based NAb assay is appropriate, it is important to study the performance parameters of the critical components in the assay system, namely the cell line, drug product, positive control (PC) NAb, and test species matrix. Cell-based NAb assays can be technically challenging because of the need to optimize the cell line, culture conditions, and sample matrix components. Lack of optimization can compromise assay precision, robustness, and sensitivity. Initially, the cells used for the drug's biological potency assay could be evaluated for use in the NAb assay. Use of the same cell line provides several advantages in that cell line maintenance and culture conditions have been optimized. Often, this system can be adapted for a biological matrix. Alternatively, commercial sources of cell lines are available, or a drug-responsive cell line could be genetically engineered. Cell lines transfected with target receptors can offer the advantage of more controlled receptor density, specificity, and enhanced signaling. Reporter gene expression cell lines can provide sensitive assay endpoints.

The cells' responsiveness to the drug should be characterized, particularly before and after freezing, as well as during continuous culture (see also general information chapter *Cryopreservation of Cells* ⟨1044⟩). Drug dose-response experiments in the appropriate biological matrix (e.g., serum) can identify early interference issues. Ideally, several matrix samples should be evaluated. Specificity should be studied to allow use with serum from the species of interest that developed NAb after exposure to the drug. PC antibodies are often isolated from antisera of this species, but because this is not always available during assay development, a surrogate control antibody reagent is produced to use during assay development and validation (see the section *Development of Positive and Negative Controls in NAb Assays*). Cell-based assay formats that have been used successfully in

drug product development are shown in *Table 1*. The type of assay format chosen is influenced by the mechanism by which the drug interacts with cells. These interactions can either be direct or indirect. A direct interaction is one in which the drug product exerts its effect by acting directly on cells, such as with cytokines, peptides, or monoclonal antibodies to cell surface determinants. Indirect interactions are those where the drug product blocks the interaction of a ligand with its cell surface receptor and consequently interferes with the biologic activity of the ligand. Examples of such drugs include therapeutic monoclonal antibodies to soluble factors and soluble receptors.

Table 1. Frequently Used Cell-Based NAb Assay Formats

Assay Endpoint	Assay Platform Examples	NAb Action	Advantages	Disadvantages
Cell surface interactions	Fluorescence cytometry, plate-based cell surface binding	Interferes with binding of drug to target site on the cell, or affects internalization of drug product into the cell.	Drug, target, and NAb interaction occurs only on the surface of cells, which may result in simple and robust assays.	Does not apply to drugs with a MoA involving signaling pathways within cells; inadequate receptor expression on cell may be a limitation.
Phosphorylation of intracellular substrates	Kinase receptor activation, enzyme-linked immunosorbent assays (ELISAs), intracellular staining	Affects drug-induced phosphorylation of target receptor/substrate, or affects ligand-induced phosphorylation by blocking drug.	Utilizes early event in cell signaling, which may result in a shorter assay.	Needs phosphorylation site-specific antibodies; multiple assay steps are needed if using ELISA to detect phosphorylation.
Cell proliferation	^{3}H incorporation, Alamar Blue	Affects cell proliferation that is induced or inhibited by drug, or affects ligand-induced cell proliferation by blocking drug.	Well-established assay endpoint for growth factor-type drugs reflects what happens to the cells.	Assay endpoint is the outcome of multiple intracellular pathways and steps, which may result in a long assay; prone to interference by serum factors; assay needs to show specificity.
Reporter gene expression	Luciferase, β-galactosidase, chloramphenicol transacetylase	Affects reporter gene expression by drug product, or affects ligand-induced reporter gene expression by blocking drug.	Assay can be quick, sensitive, and robust.	Need to invest time to construct reporter gene cell line that can respond robustly in presence of test species serum; need to demonstrate specificity.
Protein expression or secretion	ELISA, enzyme immunoassay, radioimmunoassay, electrochemiluminescent assay (ECL), scintillation proximity assays	Influences protein expression by drug, or influences ligand-induced protein expression by blocking drug.	The protein can be measured in the cell supernatant or in cell lysate by using an ELISA.	Assay endpoint is the outcome of multiple intracellular pathways and steps, which may result in a long assay; assay involves cell culture followed by ligand binding assay to detect synthesized protein, thus two assays are required.
Effector functions	Complement-dependent cytotoxicity (CDC), ADCC, Fcγ receptor binding	Influences target cell fate mediated by effector functions of therapeutic antibodies.	Required for antibody therapeutics where both Fab and Fc domains are involved in function.	Multiple effector functions (ADCC, CDC, phagocytosis, etc.) may be involved, which can make assay choice, assay development, and interpretation of results challenging.
Cytotoxicity/apoptosis	Luminescent cell viability assays, terminal deoxynucleotidyl transferase dUTP nick end labeling assay	Affects cell cytotoxicity/ apoptosis induced by drug, or affects ligand-induced cell cytotoxicity/ apoptosis by blocking drug.	Directly reflects MoA of cell killing mediated by therapeutics.	Needs to demonstrate specificity.

Consideration is given to the assay endpoint, which can reflect either early or late cellular responses. Early responses include the initial binding to the cell surface, internalization, an early event in cell signaling, or gene expression. Late responses reflect the outcome of multiple intracellular pathways that result in a biological outcome such as proliferation, induction of measurable products, apoptosis, or effector functions such as ADCC or CDC.

Care should be taken to optimize the concentrations of critical assay components that influence its sensitivity for detecting NAbs. In the case of a direct NAb assay, the dose response at several concentrations of the drug product should be evaluated. The amount of drug product used in the assay should be within the linear portion of the dose-response curve that produces a reproducible response (e.g., often a 30%–80% maximal assay response). The sensitivity of an indirect NAb assay depends on both the ligand concentration and the drug product concentration.

In all cases, the appropriate controls should be incorporated. For example, in a direct assay format in which the drug acts directly on the cells to induce a biological response, the assay controls should include cells alone, cells with drug product, and cells with drug product and PC NAb. An indirect assay format should include cells alone, cells with ligand, cells with ligand and drug product, and cells with ligand, drug product, and PC NAb. The assay can be set up as a single dilution to provide qualitative information (positive or negative), as is generally the case for preclinical studies, or can use multiple dilutions to give a quasi-quantitative readout, such as the titer. Designing well-controlled assays facilitates monitoring of assay performance over time and identification of factors to consider as root causes of assay failure.

Non-Cell-Based Methods for NAb Assessment

Cell-based functional assay formats have traditionally been recommended by the regulatory agencies for NAb assessment. However, in comparison to the technical challenges of cell-based assays described above, non-cell-based immunoassays are capable of overcoming some of the technical limitations inherent to the cell-based bioassays, and have therefore become another useful technology platform for NAb evaluation. Non-cell-based immunoassay platforms, especially competitive ligand binding assays (CLBAs) when relevant to the MoA of the drug, can be used for NAb assessment if proven to specifically detect NAbs (*Figure 2A* and *Figure 2B*).

For a range of biological therapeutics (e.g., antagonistic MAb therapeutics against a soluble ligand), the drug exerts its pharmaceutical effect by binding to its target and blocking the interaction of the ligand with its cell surface receptor; consequently, the drug interferes with the biological activity of the ligand. In this instance, a non-cell-based assay method is appropriate for NAb assessment because the assay method reflects the MoA of the drug by measuring drug binding to its target and inhibition of such binding activity by NAbs.

The CLBA-based NAb assay design is based on the competition between the ligand and NAbs for a limited number of binding sites on the ligand binding therapeutic. As the level of NAb increases, less ligand binds to the drug and the measured assay response decreases when compared to a control sample that does not contain any neutralizing activity. Therefore, within the linear range of the assay, the NAb concentration is proportional to the percentage change in assay response. In theory, any ligand binding assays, such as solid- or liquid-phase immunoassays (e.g., radioligand, ELISAs, chemiluminescence, and ECL assays), radioimmunoprecipitation assays (RIPA), and SPR, may be adapted to the CLBA format for the detection of NAb in test samples (see *Appendix* and *Immunological Test Methods—General Considerations* ⟨1102⟩ and *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩, and ⟨1105⟩ for more information).

Two assay formats are available for non-cell-based CLBA (*Figure 2A* for direct format and *Figure 2B* for indirect format).

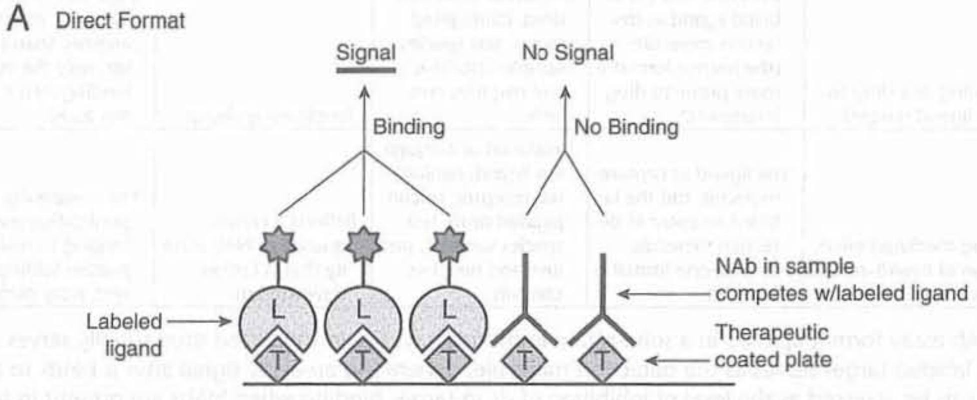


Figure 2A. Direct CLBA format: In this assay format example, the therapeutic product is coated on a plate and serves as a capture molecule to bind to the ligand labeled with a detection molecule (e.g., an enzyme, a fluorescent label, or an ECL label). Binding between the therapeutic and the ligand is inhibited when NAb is present in test samples, resulting in a lower signal. "T" represents "therapeutic product" and "L" represents "ligand."

General Chapters

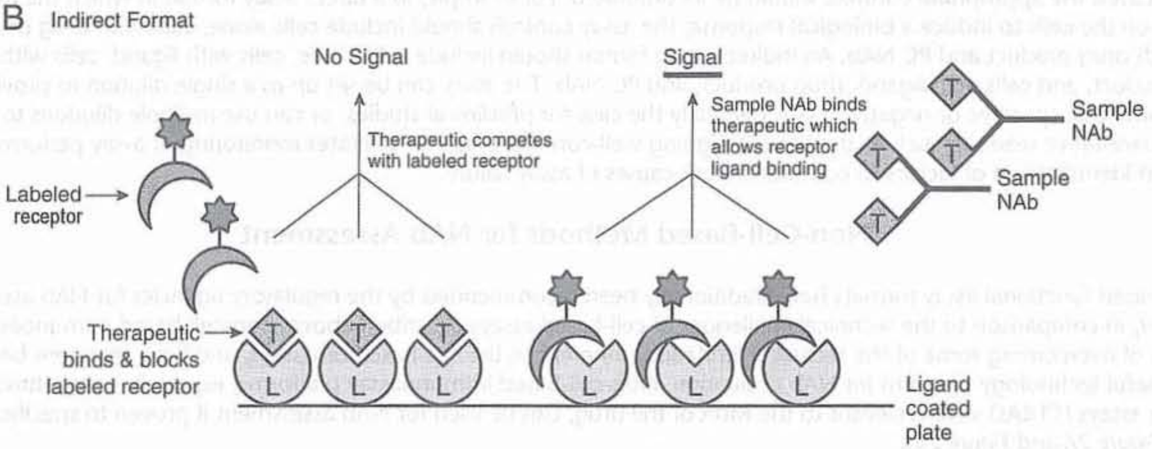


Figure 2B. Indirect CLBA format: In this assay format example, the ligand is coated on the plate, and the therapeutic competes with the labeled receptor for binding to the ligand. When NAb is present in test samples, it binds to the therapeutic and the neutralized therapeutic is unable to bind to the ligand; therefore, the signal will increase because the labeled receptor is now able to access and bind to the ligand.

The direct CLBA NAb assay format, based on measuring the binding of a drug to its target, is a simple approach (*Figure 2A* and *Table 2*), whereas the indirect CLBA NAb assay format monitors drug-mediated inhibition of ligand–receptor binding (*Figure 2B* and *Table 2*). The two assay formats both utilize the MoA of the drug (inhibition of drug–ligand or ligand–receptor binding) but measure the neutralizing activity using different assay endpoints.

Table 2. CLBA NAb Assay Format and Critical Assay Components

Assay Format	Assay Measurement	Assay Design for Solid-Phase Immunoassays	Assay Components	Advantage	Disadvantage
Direct CLBA	Binding of a drug to its ligand (target)	The drug as capture molecule and the labeled ligand as detection molecule (the reverse format is more prone to drug interference)	Unlabeled or labeled drug, conjugated target, test species samples, positive and negative controls	Simple assay design	If the binding prevents signaling or attachment of another ligand to its receptor, only the very early binding step is reflected in this assay.
Indirect CLBA	Drug-mediated inhibition of ligand–receptor binding	The ligand as capture molecule and the labeled receptor as detection molecule (the reverse format is feasible)	Unlabeled or conjugated ligand, conjugated receptor, unconjugated drug, test species samples, positive and negative controls	Reflects a consequence of NAb activity that is further downstream.	The complexity of receptor purification makes it challenging to maintain proper protein folding and consistent assay performance.

For the direct CLBA NAb assay format applied in a solid-phase immunoassay, the immobilized drug usually serves as the capture molecule, while the labeled target serves as the detection molecule, generating an assay signal after it binds to the drug. The neutralizing activity can be assessed as the level of inhibition of drug-target binding when NAbs are present in test samples. The assay sensitivity of the direct CLBA NAb assay format is largely determined by the drug concentration selected. A lower drug concentration typically leads to a more sensitive NAb assay, but a low dynamic range may limit the detection of a broad range of NAbs. Therefore, it is important to optimize the drug concentration during assay development. To further optimize the assay, implementation of design of experiment (DoE) can be considered to systematically assess the interactions among key assay-operation parameters and identify the most optimal assay conditions.

In certain cases, such as when the ligand or receptor is a highly charged molecule and tends to bind nonspecifically to surfaces, the assay format also can be reversed by using the ligand or receptor protein as the capture molecule and the drug as the detection molecule. However, because of the use of the ligand as a coating agent, this reverse format can be much more prone to drug interference even if comparable assay sensitivity is achieved. Therefore, the direct CLBA NAb assay format using the drug as the capture molecule and the conjugated ligand as the detection molecule is usually preferred.

An indirect CLBA NAb assay format, which is based on drug-mediated inhibition of ligand–receptor binding, also can be used for the detection of NAb to antagonistic therapeutics that neutralize soluble ligands. One applicable format for an indirect CLBA NAb assay uses the ligand as the capture molecule and the conjugated receptor protein as the detection molecule in a solid-phase immunoassay. The reverse format also may be feasible. The immobilized ligand binds to the conjugated receptor, generating an assay signal. Ligand–receptor binding is inhibited in the presence of drug, but occurs when the drug is neu-

tralized by NAb. The neutralizing activity in the test sample is thereby estimated by the level of restoration of ligand–receptor binding when drug function is blocked by NAb.

The sensitivity of the indirect CLBA NAb assay is largely dependent on the concentrations of both the ligand and drug. A lower ligand concentration will result in a lower drug concentration selected for the assay, leading to a higher NAb assay sensitivity. Other operational parameters also can be effectively evaluated through the DoE approach to determine the optimal assay conditions.

When an oligomeric receptor protein is included in the non-cell-based NAb assay, specific consideration should be given to the challenges presented by this type of receptor, which may be less likely to retain the original conformation necessary to bind to the ligand when not associated with the cellular membrane. In addition, cellular receptors typically have one or multiple transmembrane domains that need to be truncated to facilitate receptor purification. Therefore, concerns arise regarding proper protein folding of these truncated ectodomains and retention of the structure necessary for ligand–receptor binding. The complexity of receptor purification further requires that lot-to-lot variation and stability of the protein products be effectively managed in order to maintain consistent assay performance.

Positive and negative controls are critical for monitoring NAb assay performance. Unlike cell-based NAb assays, the design of assay controls for non-cell-based CLBA assays is usually more straightforward, without the need to include background controls. The selection of positive and negative controls is described in detail in the section *Development of Positive and Negative Controls in NAb Assays.*

VALIDATION OF NAb ASSAYS

As described above, there are two main formats for NAb assays: cell-based functional assays and binding-based assays. The various components of validation that should be carried out prior to study initiation are described in the sections that follow. The principles of each aspect of validation apply to both assay formats unless noted otherwise.

Minimum Required Dilution

Determination of the appropriate dilution of assay matrix is an important part of NAb assay optimization because this will affect the minimal test sample dilution and therefore the assay sensitivity. The considerations for defining the minimum-required dilution (MRD) used for the ADA screening immunoassay described in 〈1106〉 also may be applied to NAb assays. The effect of sample matrix on assay capability should be evaluated at multiple dilutions, preferably by using different pools of the test species matrix. The dilution of sample matrix that has a minimal effect on the assay response should be selected and further evaluated using the PC antibody spiked into sample matrix. A NAb assay should be able to detect antibodies in the presence of assay matrix components that may be expected to be present. These might include complement, coagulation factors, soluble targets, lipids, concomitant medications, and the endogenous homologous counterpart, as well as the administered drug product. As described in 〈1106〉, the MRD can be objectively determined and defined as a dilution level that achieves an optimal signal-to-background ratio with acceptable variability.

Factors such as soluble targets that either bind the drug product or act directly on the cells can interfere in the assay, leading to false positive results. Alternatively, certain factors may alter the assay response in a manner that masks the presence of a NAb in the sample (e.g., as noted with the presence of interfering levels of biotherapeutics or growth factors). Confirmation of assay specificity can help to determine the presence of such interfering substances (see the section *Assay Specificity*). Assessment of matrix effects during assay development can be accomplished using pooled matrix. Ideally, the assay matrix should be defined using multiple individual matrix samples because of heterogeneity of the various possible interfering substances. Diseased-state (treatment-naive) matrix may contain additional factors that interfere in the assay and should be evaluated whenever possible.

If lipemic, hemolyzed, incompletely clotted, and, preferably, disease-state sera from naive subjects are available, they should be screened in the assay both with and without the addition of the PC NAb. If the unspiked sera give a response in the assay, and/or the sera interfere in the detection of the control, the analytical procedure should state that samples compromised in such a manner may not yield reliable results and may have to be excluded from testing.

Development of Positive and Negative Controls in NAb Assays

NAb assays, including cell-based functional assays and non-cell-based immunoassays, are designed to detect heterogeneous and often polyclonal anti-drug immunoglobulins. Because of the diverse nature of immune responses to the drug, it is not possible to generate a true NAb reference standard. Assay performance is therefore monitored by utilizing surrogate positive and negative controls (PCs and NCs). Typically, an NC sample is generated by pooling relevant matrix that is negative for ADA, such as matrix collected from subjects with no previous exposure to the drug. One should consider whether the NC pool can appropriately represent the target study population, such as by comparing assay response produced by the NC to the mean response produced by the individual target population matrix samples (at least 20 individuals). In some cases, it can be difficult to obtain a sufficient number of study population-relevant matrix samples or to ensure that samples used in assay valida-

tion accurately represent matrix characteristics of the study population samples. The incurred study baseline samples should be assessed for any evidence of predose reactivity to the compound.

NAb assay PCs typically consist of a hyperimmunized animal serum, a monoclonal Ab, or material that otherwise has specific, and generally high, affinity neutralizing ADA reactivity. The antibody preparation used to generate a NAb-assay PC must be able to neutralize the biological activity of the drug compound in vitro. Ideally, the PC may be prepared by spiking immunoaffinity-purified ADA (polyclonal or monoclonal), or a protein-A/G purified preparation spiked into an appropriate neat matrix may be used. Alternatively, for preclinical studies only, whole hyperimmunized animal serum spiked into appropriate neat matrix can be used. For MAb biotherapeutics, the PC generally has anti-idiotypic antibody reactivity. The PC antibody is considered a critical reagent and should be documented and characterized for use in the assay. Pertinent documentation and evaluations that should be considered include immunization scheme, purification procedure and yield, protein concentration, relative affinity, cross-reactivity, isotype, and NAb titer. Thorough characterization will promote reagent consistency over time and minimize lot-to-lot variation. Refer to 〈1106〉 for stability monitoring of assay controls.

Although PCs are used to develop, validate, and monitor performance of NAb assays, these controls are not assumed to represent actual incurred study samples. A great diversity of immune responses to drug molecule, with a broad range of binding affinity and specificity characteristics, should be expected. Therefore, PC and NC performance is used primarily to ensure that the assay performs as expected (i.e., to confirm system suitability).

Assay Cut-Points

The cut-point of a NAb assay is the assay response used to determine whether a sample is positive for neutralizing activity. All individual drug-naive subject samples and NC samples used for the cut-point evaluation should be spiked with a fixed concentration of drug determined prior to validation; however, this would not apply to the direct format for non-cell-based NAb assays. The assay response can be expressed as assay signal or as the ratio of assay signal from a test sample to that derived from the NC. The NAb-positive samples would have a response above the cut-point if NAb increases the assay response and below the cut-point if NAb reduces the assay response. Alternatively, the assay response may be normalized and computed as percentage change from the NC.

Because 〈1106〉 includes an outline of the cut-point evaluation process, this chapter only provides a summary of the evaluation process, with emphasis on and clarification of some key steps for NAb assays (additional helpful resources can be found in the *Appendix*). Alternative statistical methods can be applied in some steps of the cut-point evaluation process (e.g., outlier evaluation) with appropriate justification.

Based on statistical and practical considerations, at least 30 individual subject sera from the target disease population (if available) or healthy donors should be used for cut-point evaluation. Samples from these subjects should be tested over at least three independent runs (e.g., days, analysts) by at least two analysts using a balanced-design framework. At least three reportable results for the negative control should be available from each plate, where a reportable result may be the average of duplicate sample results if the study samples are also tested in duplicate. Also, these three results should come from different locations in the plate, such as the first column, middle column, and last column of the plate.

If the cut-point is estimated from only healthy donor sera during this validation phase, data from the target disease population should be evaluated during the in-study phase to determine statistically whether the distributions of assay response are similar to those of the healthy population. If the variances are significantly different, the cut-point should be re-evaluated using the target disease population. If only the means are significantly different, the same cut-point can be used after redefining the NC based on the target population. Other population differences relevant to the clinical study with respect to gender, age, and other factors also should be considered in the sample selection process.

The distributions of original data and log-transformed data (or other transformations), averaged across assay runs, can be evaluated in terms of the skewness coefficient and normal probability plot. Data from the scale (e.g., original, log) that provides the most symmetric or close-to-normal distribution should be used in all subsequent analyses, such as outlier evaluation, cut-point calculations, and comparisons of means and variances across assay runs. Analytical and biological outliers should be identified using appropriate statistical methods such as using the conditional residuals and subject mean estimates from a mixed-effects model that includes the relevant sources of variation in the cut-point experiment (e.g., this model may include Subjects nested within Subject Groups, Run Number nested within Analyst, and Plate ID as random-effects, as well as Subject Groups, Analyst, Plate Testing Order, and the interaction of Analyst and Plate Testing Order as fixed-effects).

The analytical outliers should be identified and removed before identifying the biological outliers, and the distribution of assay response should be evaluated after removing the analytical and biological outliers. If the distribution is adequately normal (i.e., Shapiro–Wilk test is not significant), the parametric approach [mean +2.33 × standard deviation (SD), if NAb increases the assay response, and mean −2.33 × SD, if NAb reduces the assay response] can be used for determining the cut-point from these validation data. This calculation will yield a 1% untreated false positive rate from a normal distribution. The cut-point may be defined in terms of other false positive rates, such as 0.1%–5% on a case-by-case basis, with appropriate justification and discussions with regulatory agencies. If the distribution is not adequately normal (i.e., Shapiro–Wilk test is significant) but is still symmetric enough (skewness coefficient <1), and the departure from normality is mostly due to the heavy tails or some extreme values that may not have been identified as statistical outliers, robust alternatives to the mean and SD, such as the median and 1.4826 times the median absolute deviation, respectively, can be used in the above cut-point calculation formula.

If the distribution is highly nonsymmetric (skewness coefficient >1), the nonparametric 99th percentile is recommended, although this should be a last resort as it requires a much larger number of subjects (>100) to obtain a reliable estimate.

Another important consideration is that the SD estimate used in the cut-point estimation should include all the different sources of variation that are relevant to the context where study samples will be tested during the in-study phase. Because study samples are typically tested by multiple analysts over several plates and assay runs, the SD used in the cut-point evaluation in such cases should include, at the minimum, interanalyst, interplate/run, intraplate/run, and intersubject variability.

In order to understand the nature of variability in the assay, and to determine whether the same cut-point evaluated during the validation phase can be used for identifying samples with neutralizing activity during the in-study phase, the means and variances of the distribution of the assay signal from approximately six runs should be compared using a mixed effects model and Levene's test, respectively (see ⟨1106⟩). If these are not significantly different, then the same cut-point (fixed cut-point) can be used during the in-study phase. Otherwise, the cut-point evaluated as above using these validation data should be divided by (or subtracted from) the NC. This is called a multiplicative (or additive) normalization factor. This factor may be multiplied (or added) to the NC used during each run of the bioanalysis phase to define the run- or plate-specific cut-point. Such a cut-point is called the floating cut-point. If the original data are found to be approximately symmetric or normal, then an additive correction factor may be used. If a log transformation is necessary to ensure approximate symmetry or normality of the distribution or if the distribution of the ratio of original sample results (assay signal) to NC has been shown to be adequately symmetric or normal, then a multiplicative correction factor may be used. In such cases, all of the analyses for cut-point evaluation also may be done in terms of the ratio of assay signal from the individual subject sample to the NC from the corresponding plate.

In practice, regardless of whether the means and variances are significantly different between assay plates or runs, the use of a floating cut-point is recommended as it is more accommodating to minor drifts between assay runs during the in-study sample testing phase.

If a fixed cut-point is justified from the above evaluations and therefore is implemented during the in-study phase, a higher level of attention is required to monitor and validate changes in reagents and other assay conditions. Because the cut-point has been fixed in relation to that assay as it existed at the time of the experiments that determined the cut-point, it is essential to ensure that the assay signal results from the controls and test samples are consistent and stable in the event that any changes are made to the assay (e.g., new reagents, analysts, or machinery).

System Suitability Criteria

NAb assays typically have an intricate design with multistep operations. These assays use complex reagents and equipment and also require extensive data collection. It is therefore important to conduct system suitability assessments in order to evaluate and verify overall method validity and utility.

Specifically, negative and positive assay controls and additional background controls (e.g., cells alone and/or cells with drug product) should be included as part of each analytical run during assay validation and during the sample testing phase. The exact nature of background controls will depend on the type of NAb assay developed (see the section *Design of NAb Test Methods*). Data obtained during assay validation are used to develop assay acceptance criteria.

Generally, monitoring performance of the PC at low level (LPC) and high level (HPC) is most critical. Inclusion of the LPC helps monitor the established assay sensitivity. Exclusive use of a mid-level PC (MPC) should be avoided because the middle range of the assay response versus PC concentration may not be affected as significantly by changes in assay conditions (e.g., reagent, changes) as are LPC and HPC. Alternatively, performing an analysis of a PC tested in a dilution series provides coverage of multiple PC levels, including LPC. In some circumstances, use of an NC that includes a non-neutralizing antibody may be helpful for identifying run failure and preventing reports of false positive results. Typical acceptance criteria for the assay control samples include (a) precision of the raw assay signal for PC and NC samples, (b) performance of the LPC sample or reportable titer value for the PC, and (c) upper and/or low limit for the assay response generated by the assay NC. Other criteria may be applied.

When the floating cut-point approach is used for the NAb assay cut-point evaluation, the system suitability criteria or limits can be defined for the ratio of the LPC to the NC and for the ratio of the HPC to the LPC, instead of defining limits separately for each PC. For the in-study phase, it is also useful to apply acceptance criteria for intra-assay precision (variability of response of replicates in an assay). As discussed in ⟨1106⟩, setting criteria for passing or failing assays in pre-study validation experiments should be avoided and all assays performed during pre-study validation should be included except for those rejected for an assignable cause. The appropriate choice of LPC concentration and the acceptable range of performance are important for ensuring the ability to monitor sustained assay sensitivity. The failure rate for LPC is expected to be 1% based on the PC performance. Similarly, an assay failure rate can be set at 1% based on the performance of the NC. If applicable, higher rates of failure (e.g., 5%) may be used. To set a specific failure rate based on the NC and LPC sample performance, appropriate assay response limits should be calculated based on control performance during assay pre-study validation. Alternatively, limits may be established for the PC titer value. These should be defined based on the PC performance observed during the pre-study assay validation phase. Importantly, depending on the specifics of the NAb assay format, the signal in the assay is expected to increase (*Figure 3A*) or decrease (*Figure 3B*) in response to the increasing PC concentration. Hence, an appropriate NC limit should be set. For example, in the first case, where the assay signal is increased, an upper limit for the NC should be established. In the second case, a lower limit for the NC will be most important.

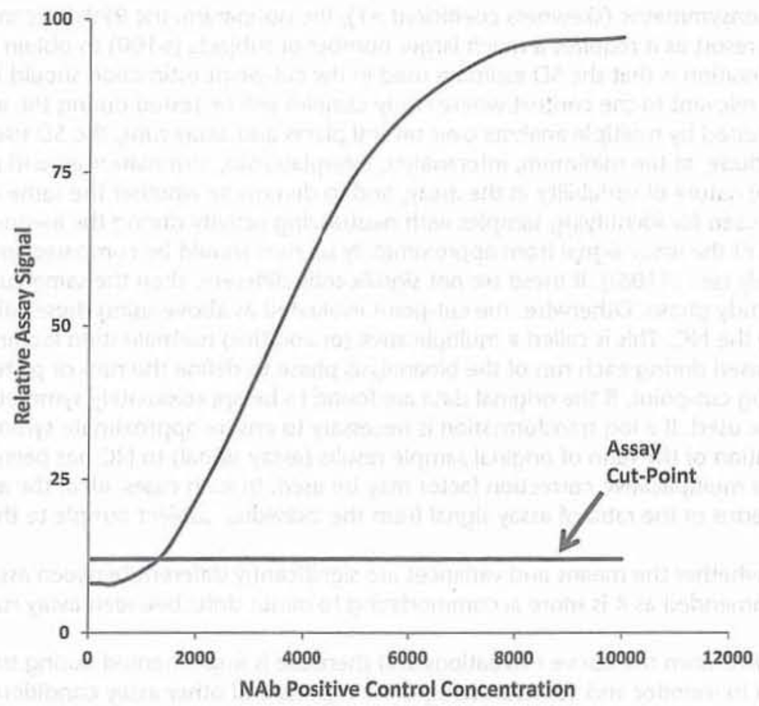


Figure 3A. NAb assay response as a function of PC concentration. Assay signal increases with increasing PC concentrations.

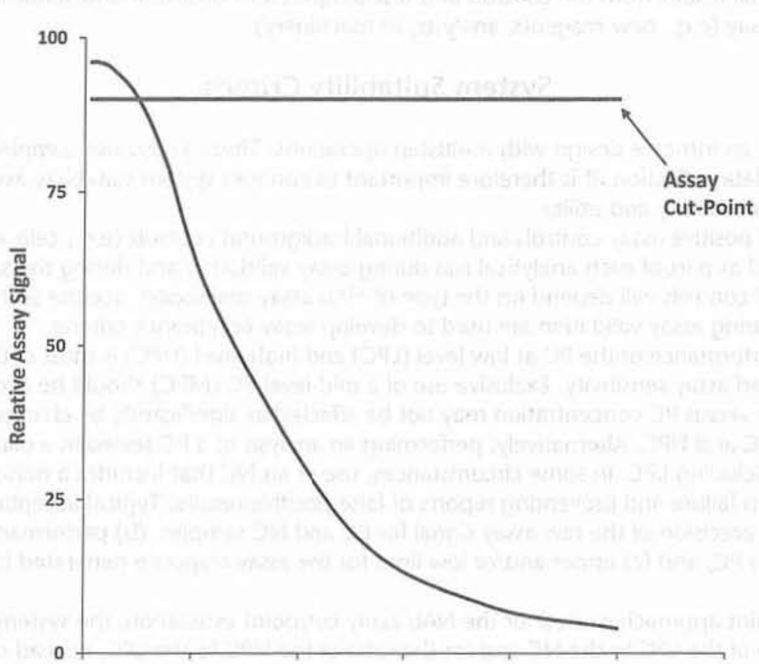


Figure 3B. NAb assay response as a function of PC concentration. Assay signal decreases with increasing PC concentrations.

Initial assay control acceptance criteria may be based on the existing information about assay performance obtained during the assay development and qualification phase. Alternatively, standard criteria that are based on the specific, existing NAb assay validation standard operating procedure may apply. Important technical details such as instrument signal readout capabilities should be considered when setting expectations for the assay control performance. Actual criteria for the assay controls can only be determined after holistic evaluation of the assay validation information in its entirety.

Because of the complexity of the NAb assays, specifically the cell-based NAb protocols, an effort should be made to identify particular steps and conditions with the highest potential to affect assay performance. Generally, assay control performance should be monitored during the study support phase to ensure consistency of reported data. Assay control performance data

should be monitored for any short- or long-term trends and variations relative to the predefined range. If a suspicious trend in the control sample performance is identified or the signal falls close to a limit of the acceptable range, an investigation should be considered.

Relative Sensitivity

NAb assay sensitivity is defined as the lowest concentration of an assay PC antibody that can be detected reliably by the method. The result obtained for this validation parameter is highly dependent on the characteristics of the PC used to conduct the experiments, including its neutralizing capacity and binding affinity to the drug. The relative sensitivity of NAb assays is also inversely dependent on the concentration of drug used in the assay.

To conduct the experiment, PC is spiked into assay-relevant pooled matrix. These spiked samples are tested in multiple runs, commonly in at least three independent runs by two operators for a total of six runs. It is recommended that more than one antibody curve be performed per run per operator.

Generally, linear interpolation between values just above and below the assay cut-point is conducted to calculate the assay sensitivity parameter. In some cases, a four-parameter model is used for which at least six values generated by various concentrations of PC should be available to appropriately analyze the resulting data set. One of the values should fall below the assay cut-point. When using linear interpolation or a four-parameter-fit approach, the PC concentration that would generate an assay response equal to the cut-point value is calculated. Values generated in multiple tests over multiple days are averaged, and the result is reported as the relative sensitivity of the assay. Once established, the assay sensitivity value can be used to guide selection of a LPC concentration to be used when monitoring assay performance during assay validation and when testing incurred study samples. Generally, the LPC concentration is chosen so that the rate of assay failure due to performance of the PC is NMT 5%.

Because the assay sensitivity depends greatly on the characteristics of the assay PC, assay sensitivity will vary between different NAbs. The assay sensitivity value determined during assay validation cannot be used to predict the actual NAb concentration that could be detected in study samples. The NAb assay sensitivity parameter is useful when evaluating various analytical platforms, during assay development and optimization, and for selecting appropriate PC concentrations for assay validation and system suitability testing. Typical targets for the NAb assay sensitivity are often 0.5–2 µg/mL; however, because NAb assays are complex, a case-by-case, fit-for-purpose approach to selecting an appropriate assay sensitivity is used with the goal of detecting clinically relevant NAbs.

Assay Specificity

Assay specificity is defined as the ability to unambiguously detect the analyte of interest. For example, in the case of cell-based assays, an initial assessment should be done to investigate the ability of the chosen cell line to respond to biological components expected to be present in the assay matrix that may structurally or functionally resemble the drug molecule or its molecular target; the goal is to ensure the specificity of the NAb format. Such an assessment may aid in determining the assay format, the analytical platform, the type of cell line to be used in the assay, any sample pretreatment, and other assay conditions. Careful attention should be paid to potential differences between normal and disease-state samples. Assessment of assay specificity may include the testing of irrelevant ADA (e.g., antibodies to other similar molecules) and should include, where possible, specific binding ADA that are known to lack neutralizing activity. The comparison may be made either by evaluating an antibody dilution profile or by spiking the antibody in excess into a pooled negative matrix sample. The irrelevant antibodies are not expected to score positive in a well-designed, specific NAb assay. Other matrix components (e.g., soluble forms of receptors or other drug binding partners) may exhibit inhibitory effects on the drug activity. It is important to understand whether true confirmatory testing should be included as part of an incurred sample analysis routine. Such confirmatory testing should demonstrate that the inhibition of the drug activity is specific to NAbs and not other factors found in the assay matrix. NAb confirmatory testing, also referred to as matrix interference assays, generally employ a cut-point-based approach. If a decision is made to include a matrix interference NAb confirmatory assay during routine sample testing, the validation should follow the general principles described above for the assay cut-point determination.

Selectivity and Interference

Selectivity evaluates the ability of a NAb assay to detect a NAb PC in a matrix sample containing potential interfering factors. These matrix factors may bind to NAb through specific or nonspecific interactions, interfering with NAb detection. General matrix interference can be investigated by evaluating the recovery of the NAb assay response generated by the HPC and LPC prepared in 10–20 individual relevant matrix samples.

One of the major interfering agents in a NAb assay is the drug itself, when present in test samples from dosed subjects. The drug interferes with the ability of the assay to detect NAbs, causing false negative or false positive results, depending on assay design. The magnitude of drug interference is dependent on multiple factors such as the circulating drug concentration, the concentration and other characteristics (affinity, avidity) of the PC antibody, the half-life of the drug, and the assay design. Therefore, it is not possible to establish a universal "drug tolerance level" for all NAb assays. Nevertheless, drug interference in

the NAb assay should be addressed in the assay design and testing strategies. During assay development or optimization, drug interference may be assessed by adding titrated concentrations of drug into undiluted matrix containing fixed concentrations of a positive NAb control, based on assay sensitivity. The limit of drug tolerance is reported as the highest concentration of drug at which PC NAb remains detectable, where detectability should be defined (e.g., a certain signal-to-noise ratio, a selected level above background). To ensure that the assay method is sensitive enough to detect NAbs in the presence of circulating drug, the positive NAb control could be titrated in undiluted pooled matrix sample (e.g., at 250 ng/mL or 500 ng/mL for clinical studies or 1000 ng/mL for nonclinical studies) to assess drug tolerance level for the assay method. Thus, based on the sensitivity of the NAb assay, the PC should be diluted to that level when trying to detect NAb in the presence of circulating drug. The drug tolerance level can vary considerably when different concentrations of the positive NAb control are added to the assay matrix. NAbs may also differ in affinity and/or avidity. Therefore, the drug tolerance level assessed using a PC may not predict the actual levels of drug interference in study samples.

Because NAb assays tend to be highly susceptible to drug interference, it is generally not recommended to test for NAbs in samples from time points when drug levels are expected to be high. However, it may be necessary to analyze NAb activity in study samples containing circulating drug to investigate when the onset of the NAb response occurs and its impact on drug exposure. Under these circumstances, methods need to be applied to overcome drug interference and enable NAb detection in the presence of high levels of circulating drug. Strategies commonly used to improve drug tolerance level include acid dissociation and removal of excess drug through physical separation or solid-phase absorption. An alternate approach is a drug quantitation-based approach in which samples are tested for the bioactivity of circulating or exogenously added drug. Each method has its own caveat. For example, acid pretreatment may affect the activity of NAbs. In cell-based NAb assays, excess acid contained in samples may negatively affect the cellular response and decrease the assay signal, thereby compromising the advantage offered by using the acid dissociation procedure.

In addition to drug interference, soluble drug ligands may also interfere with NAb assays, potentially generating false positive results. Target interference also can be introduced when the drug target is released from drug–target complexes during acid dissociation. These interfering factors may be addressed by optimizing sample pretreatment methods. One feasible approach is to pretreat the samples with beads conjugated to an anti-target antibody. After removal of beads, the target-depleted samples can be used in the NAb assay.

Precision

Precision—intra-assay and interassay—is the quantitative expression of variability, and it provides a measure of the amount of random error that occurs during the execution of an analytical procedure. Precision estimates are useful indicators of assay performance in the specified assay matrix.

QUALITATIVE NAb ASSAYS

Per general information chapter *Validation of Compendial Procedures* 〈1225〉 and ICH Q2(R1), intra-assay precision (repeatability) is the degree of agreement between results generated by consecutive analysis (replicate testing) of the same assay controls or samples under the same operating conditions, by the same operator using the same equipment in a laboratory, within a short period of time. Four to six independent preparations of LPC, HPC, and/or NC samples in a single lot of pooled donor serum (normal or disease state) are evaluated in duplicate or triplicate, in multiple positions on the same plate in a randomized manner, to determine the relevant sources contributing to response variability. The imprecision of the assay response (optical density, fluorescence unit, luminescence unit, or percentage change in assay signal after normalization or interpolation), is calculated and reported as percent coefficient of variation (%CV), which equals (SD/mean) × 100. The %CV values of the mean assay response obtained with the various assay controls should suffice for assessment of intra-assay precision. The %CV may vary depending on the technology used for detection, assay methodology, and procedural complexity. The expected target CV or pooled %CV for intra-assay precision should therefore be defined based on assay capability, as well as on intended use. It is not possible to generalize acceptable precision as it depends on the use of the assay and drug type, risk to patient, and other factors, but the need to rely on the assay result should drive acceptable limits.

Interassay precision (also called intermediate or total precision) encompasses within-laboratory variation among assay runs, and therefore represents the overall precision of the assay. The experiment described above should be executed over multiple days with at least two operators, especially if the sample testing will be executed by more than one operator in the study phase. The pooled intraplate SD and the SD of the mean for each sample tested on multiple plates over multiple days can be used to calculate the intermediate precision, assuming that most of the variability is attributable to plate variability and that the sample size is the same on every plate. Intermediate precision is highly dependent on the assay methodology and procedural complexity. The target intermediate precision, therefore, should be defined based on assay capability, as well as on intended use (fit-for-purpose).

Using an alternate approach, interassay precision may be assessed by deriving the mean, SD, and %CV of the NCs and PCs from all of the experiments conducted during assay development (provided that the plate location effects are negligible), with a few exclusions. The runs to be excluded are those with an assignable operator or equipment error, or with method variations introduced intentionally for robustness testing.

QUASI-QUANTITATIVE NAb ASSAYS

To assess the precision of the reported titers, operators generally use the LPC and one to two concentrations of the HPC. The HPCs (minimum of three independent preparations) should be diluted in a two- or three-fold titration series using undiluted pooled assay matrix as the diluent and then should be tested in the assay. The HPC can also be diluted in MRD matrix pool, as long as future samples are diluted in the same manner. To measure intra-assay precision, it is generally recommended that three titration curves of the low and high PC be analyzed by one operator on the same day. To measure interassay precision, three titration curves each of the low and high PC should be analyzed on a minimum of 2–3 different days by two operators. Titers are determined as a reciprocal value of the highest dilution of the PC that tests positive. Target titers can be determined and assigned to each low and high PC or can be calculated as mean values by averaging the titer values obtained for the low and high PCs in the precision assessment. Intra-assay and interassay precisions of titers are then evaluated by comparing titers obtained for individually prepared curves to the target titer assigned for the low PC and high PC, respectively. As described in ⟨1106⟩, a recommended but more rigorous approach is to use these data to define a minimum significant ratio (MSR). The calculated MSR reflects the smallest fold-change in the titer values that can be considered statistically significant ($P < 0.05$); for instance, if MSR = 5, then titers that are different by more than five-fold can be considered significant.

In general, the acceptance criterion for titer precision is that the assigned titer value should be within one dilution of the target titer in independent titration series. This, however, will depend on the method capability, the dilution level (e.g., this criterion may be suitable for a two- or three-fold serial dilution assay format but not a 10-fold serial dilution format), and the intended use of the reported titer data in the clinical setting. If using the calculated mean titer approach, occasionally the mean titer may fall in between the dilutions because it is derived from observed values from multiple analyses. In this scenario, the ±1 dilution rule needs to be modified, and titers observed for a defined PC are rounded to the nearest dilution to yield the target titer.

Robustness and Reproducibility

Robustness and reproducibility of immunogenicity assays are discussed in ⟨1106⟩, which is in harmony with ⟨1225⟩ and ICH Q2(R1).

Robustness testing should be done as part of assay optimization during assay development, if at all possible. This is because it is unlikely that during validation an analyst would specifically attempt to make changes that might routinely occur (e.g., switching lots of materials or varying incubation times within certain limits). It is therefore necessary to understand which parameters of the method require strict adherence (e.g., concentration of coating antibody), with precise limits for the parameter delineated in the method, versus parameters that need less control and can have "approximate" descriptions in the method.

Robustness experiments can be performed in a simple fashion by varying one or two variables at a time or by DoE approaches, depending on how many factors will be tested at any one time and the extent to which interactions among parameters will be examined. The breadth of robustness testing depends on the intended use of the assay (e.g., a small study looking for gross changes in a toxicology experiment might need less robustness testing than some other studies). However, any assay used for clinical studies involving the registration of a drug should be examined thoroughly to ensure that it performs as expected on a routine basis over an extended period. Plate edge effect (or uniformity) should be examined during assay development. Other common factors that should be evaluated for impact include incubation times and temperatures, reagent concentrations, and cell densities.

If an assay needs to be transferred to a different laboratory, analysts should assess the reproducibility of the method in the new laboratory. This can be done as part of the assay transfer qualification and/or the validation of the method at the new facility. It is important to note that a properly designed and optimized assay, where critical parameters have been understood and controlled, should be robust enough to transfer without any issues. However, to ensure that the assay will perform in the same manner in each laboratory, several useful indicators can be assessed. Shared samples can be tested at both sites, and estimates of assay variability and quality control performance can be evaluated (see also the section *Transfers to Other Laboratories* in *Life Cycle Management*).

Stability

It is important to understand the optimal storage and handling conditions for assay samples, controls, materials, and reagents (see ⟨1106⟩ for additional guidance). For example, it may be important to provide advice to clinics on aliquoting procedures, time to freeze samples, storage temperature, and shipping conditions.

A key consideration for cell-based NAb assays is the stability of the cell line itself. Cell lines, as living entities, exhibit inherent variability and the potential to change over time or react to the environment in a way that can influence their response in the assay. For example, variations in levels of cell surface receptors may be affected by the number of cell doublings, time in culture, presence of certain media components (e.g., serum), and/or cell density. Therefore, during NAb assay development, cell line performance should be thoroughly characterized. Appropriate controls should be established for parameters including passage number, reagents, and media changes. An example is that the use of frozen cell aliquots of the same passage number for each assay can sometimes reduce variation because of changes in continuous culture.

General Chapters

Documentation of Pre-Study and In-Study Validation

The recommended documentation is described in chapter 〈1106〉 for both pre-study and in-study validation.

IN-STUDY VALIDATION ASSAY MONITORING

The ability of a NAb assay to perform in a reliable manner over time is important for conducting historical comparisons of NAb incidence for a single biotherapeutic as it advances through the clinical development life cycle. Also, verification that a validated assay continues to perform as expected is an ongoing process, and once the NAb assay is implemented, it is good scientific practice to monitor its performance by trending the results obtained with assay controls over time. It is recommended to use a statistical approach (e.g., Westgard rules) for assigning the threshold for assay failure and investigation based on behavior of the assay control (positive or negative). This may be easier to accomplish for highly precise assays than for assays that tend to be more variable. Regardless, it is recommended to tabulate the assay control values obtained from a minimum of 10 runs over an appropriate time period to establish a threshold and an approach for identifying assays that seem to be trending toward the assay limits. If the assay performs outside pre-established expectations, an investigation should be performed to identify the cause of the observation; implementation of appropriate step(s) may be necessary. If corrective step(s) are required, assay performance verification may be necessary to demonstrate that performance has returned to its original level. It is also important to identify variables that are likely to contribute to assay drift, such as the use of a new working cell bank, changes in lots of critical reagents, and different assay operators.

LIFE CYCLE MANAGEMENT

Changing the NAb Assay Format

During the drug development phase, it may be necessary to change the NAb assay format. For example, the assay format might be changed from cell-based to non-cell-based or vice versa to obtain an assay with better sensitivity or specificity, or other desirable characteristics. If NAb-related adverse events tend to be serious or life threatening (e.g., in the case of growth factor or cytokine drug products that have a nonredundant function), it is recommended to seek regulatory agency advice when considering a different assay format to ensure its suitability for detection of clinically relevant NAbs.

For all NAb assay format changes, an assessment of assay sensitivity and specificity is important. If the switch is being made for greater ease of performance without a significant improvement in assay characteristics, the ability of the previous and new formats to detect NAbs should be compared. This comparison should be made by using donor serum samples spiked with the PC antibody, as well as incurred study samples that have previously tested positive for NAbs. For the latter, a rate of concordance for sample results should be predetermined and met.

Transfers to Other Laboratories

NON-CLIA LABORATORIES

If the receiving laboratory is not certified under the Clinical Laboratory Improvement Amendments (CLIA) program, the capability of this lab to follow good laboratory practices (GLP) should be assessed before initiating any assay transfer activities. CLIA-certified laboratories are regulated by the Centers for Medicare and Medicaid Services of the U.S. Department of Health and Human Services. These regulations are defined in 24 CFR Part 493 and apply to laboratories testing human specimens for the purpose of disease diagnosis, prevention, monitoring, or treatment. For non-CLIA labs, the capability assessment may include evaluation of existing infrastructure for conducting cell-based or non-cell-based NAb assays, review of staff training records, and other activities. An informal feasibility study may be useful for assessing assay performance in the receiving lab. The transferring lab can simply provide the assay and reagents to the receiving lab, which can attempt a few runs and evaluate the assay controls. This exercise helps evaluate both the clarity of the written method and the receiving lab's ability to conduct the assay. The feasibility study results should be useful when determining the level of formal training that the transferring lab should provide to the receiving lab. Formal training, if needed, should adhere to a documented training plan. Assay controls and training samples prepared by spiking matrix samples with the PC NAb may be run by both the trainer and trainee following the same detailed method. The acceptance criteria for successful training should be clearly described in the training plan.

Formal assay transfer activities may commence after the receiving lab has been trained successfully and its equipment has been qualified. An assay transfer protocol should be written to describe the experiments that will be conducted during the assay transfer phase. The acceptance criteria for the experiments should be clearly defined. Some typical experiments that should be conducted by the receiving lab during assay transfer may include (a) running PC antibody curves, (b) testing matrix samples that have been left unspiked or have been spiked with PC NAb, and (c) testing different lots of critical reagents, performed by more than one analyst. For the experiments designated (b) and (c), it may be useful for both the transferring lab and receiving lab to conduct the experiments using the same training samples. Statistical analyses then should be performed on the data generated, to assess the degree of concordance between the two labs. The extent of concordance required for a

successful assay transfer should be detailed in the protocol. In certain situations, it may be necessary to derive a new assay cutpoint or a change in assay sensitivity because of a change in reagents or PC NAb. All changes to the method should be captured in a revised method supplemented by an assay transfer report that details all the experiments that support the changes. After a successful assay transfer, the receiving lab may implement the method for analyzing study samples. Appropriate assay trending approaches should be implemented to monitor assay performance and thereby ensure that it remains within recommended specifications.

CLIA LABORATORIES

For NAb assay transfers to a CLIA lab, one should follow the relevant guidelines for laboratory staff qualifications and for review and approval of the assay training and transfer documents by personnel who provide oversight to CLIA tests. The approach for NAb assay transfer to GLP (non-CLIA) labs, described above, may be used for CLIA lab transfers as well. Annual competency assessments, quality assurance monitoring and review of assay controls, reporting of individual patient test results to treating physicians, and proficiency testing are required of the CLIA-compliant laboratory. Proficiency testing (PT) should be performed twice annually. The testing may include preparation of pooled matrix samples that are tested to establish a baseline. Aliquots are then provided to the testing group in a blinded manner (at least 5 PT samples/PT event), and the results are compared to baseline. Overall, 4 of 5 samples must pass PT, and any discrepant results are investigated.

Cross-Validation and Bridging

Cross-validation may be necessary if the same assay needs to be run or maintained at multiple labs simultaneously. The first step is training of the personnel who run the assay at the new location; this is often provided by the originator lab. Several components of the assay transfer activities mentioned above may be applied during a cross-validation effort. Assay performance may be monitored using assay controls and matrix samples, which are spiked or left unspiked at both locations. If the NAb assay is altered within a study, bridging experiments should be performed. This may be accomplished by running incurred samples previously tested by the old method in the new method and assessing concordance. For a certain period of time, study samples may be run in both the old and new methods at the same time to ensure that the new method is acceptable.

Method Improvements or Changes

The laboratory should implement a process for introducing any known change in the assay that might impact assay performance. Appropriate qualification experiments should be conducted before introducing the change to ensure that it will not interfere with the historical performance of the assay. If the change requires an adjustment of the assay acceptance criteria, appropriate documentation may be required to show the impact of the adjustment on the intended use of the assay. A statistician should be consulted if needed.

For cell-based NAb assays, a change in the method might involve use of a different cell line or selection of a different assay endpoint with the same cell line to achieve improved assay characteristics. A change in the NAb assay readout platform is sometimes made (e.g., ELISA yielding absorbance values versus ECL values). In cases of method improvement, both assay development and assay validation will be needed.

CROSS-VALIDATION TO OTHER SPECIES

During the drug development cycle, there may be a need to change the assay matrix from one species to another (e.g., cynomolgus or chimp to human) or across patient populations. The change of assay matrix may require an investment in both assay development and assay validation efforts to ensure that the assay shows acceptable sensitivity, specificity, and other characteristics.

REAGENT REPLACEMENT

Reagent qualification is required if a critical reagent needs replacement using previously established qualification criteria. Ideally, the reference lot and the new lot should be compared by preparing assay controls. However, in many cases a reference lot may not be available for conducting a comparison. In this case, the new reagent should be tested and if the assay behaves as expected, it may be considered acceptable.

Assay Standardization

There is an emerging clinical need and apparent value in assay standardization (i.e., use of reference standards, platform, and reference method) to facilitate harmonization of the approach used for the detection of NAbs directed toward drugs in the same class of therapeutic [e.g., interferons, anti-tumor necrosis factor (TNF) MAbs, or erythropoiesis-stimulating agent therapies]. If several companies are involved, a consensus approach to standardizing the method should be undertaken. This

requires agreement on a universal method and protocol with a common set of reagents in all laboratories concerned. For cell-based NAb assays, establishment and implementation of a common, master cell bank is critical. The reporting units for NAb-positive samples also must be unified, such as, expressing results as positive or negative versus specific values with units and/or using a standard protocol and common reagents for calculation of NAb activity. To confirm that standardization has been implemented successfully, the universal method should be used by all labs involved in the standardization effort to analyze a set of matrix samples spiked or unspiked with NAb PC in a blinded manner. Analysis of known positive and negative study samples also should be performed.

APPENDIX: ADDITIONAL SOURCES OF INFORMATION

General

Hu J, Gupta S, Swanson SJ, Zhuang Y. A bioactive drug quantitation based approach for the detection of anti-drug neutralizing antibodies in human serum. *J Immunol Methods.* 2009;345(1-2):70–79.

Lofgren JA, Wala I, Koren E, et al. Detection of neutralizing anti-therapeutic protein antibodies in serum or plasma samples containing high levels of the therapeutic protein. *J Immunol Methods.* 2006;308(1-2):101–108.

Mire-Sluis AR, Barrett YC, Devanarayan V, et al. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods.* 2004;289(1-2):1–16.

Shankar G, Devanarayan V, Amaravadi L, et al. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal.* 2008;48:1267–1281.

Antibodies Affecting Drug Clearance

Subramanyam, M. Case study: Immunogenicity of Natalizumab. In: van de Weert M, Moller EH, editors. *Immunogenicity of biopharmaceuticals.* New York: Springer; 2009. pp. 173–187.

Zhou, L, Hoofring, SA, Wu, Y, et al. Stratification of antibody-positive subjects by antibody level reveals an impact of immunogenicity on pharmacokinetics. *AAPS J.* 2013;15(1):30–40.

Design and Validation of NAb Assays

Gupta S, Indelicato SR, Jethwa V, et al. Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods.* 2007;321(1-2):1–18.

Gupta S, Devanarayan V, Finco D, et al. Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics. *J Pharm Biomed Anal.* 2011;55(5):878–888.

Cell-Based Methods

Jolicoeur P, Tacey R. Development and validation of cell-based assays for the detection of neutralizing antibodies to drug products: a practical approach. *Bioanalysis.* 2012;4(24):2959–2970.

Tovey MG, Lallemand C. Improved analytical methods for the detection and quantification of neutralizing antibodies to biopharmaceuticals. *Bioanalysis.* 2012;4(17):2179–2190.

Wang J, Lozier J, Johnson G, et. al. Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment. *Nat Biotechnol.* 2008;26(8):901–908.

Non-Cell-Based Methods

Finco D, Baltrukonis D, Clements-Egan A, et al. Comparison of competitive ligand-binding assay and bioassay formats for the measurement of neutralizing antibodies to protein therapeutics. *J Pharm Biomed Anal.* 2011;54(2):351–358.

Thorpe R, Swanson SJ. Current methods for detecting antibodies against erythropoietin and other recombinant proteins. *Clin Diagn Lab Immunol.* 2005;12(1):28–39.

Wadhwa M, Bird C, Dilger P, et al. Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals. *J Immunol Methods.* 2003;278(1-2):1–17.

Wadhwa M, Thorpe R. Assessment of unwanted immunogenicity. In: van de Weert M, Moller EH, editors. *Immunogenicity of biopharmaceuticals.* New York: Springer; 2009. pp. 57–73.

Wu BW, Gunn GR, Shankar G. Competitive ligand-binding assays for the detection of neutralizing antibodies. In: Tovey MG, editor. *Detection and quantification of antibodies to biopharmaceuticals: practical and applied considerations.* Hoboken, NJ: John Wiley & Sons, Inc.; 2011.

Statistical Methods

Devanarayan V, Tovey MG. Cut points and performance characteristics for anti-drug antibody assays. In: Tovey MG, editor. *Detection and quantification of antibodies to biopharmaceuticals: practical and applied considerations.* Hoboken, NJ: John Wiley & Sons, Inc.; 2011.

Regulatory Guidance

EMEA. Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use. 2012. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf. Accessed 16 Sep 2013.
EMEA. Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. 2007. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003947.pdf. Accessed 16 Sep 2013.
FDA. Guidance for industry. Assay development for immunogenicity testing of therapeutic proteins. Draft guidance. 2009. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf. Accessed 16 Sep 2013.
FDA. Guidance for industry. Immunogenicity assessment for therapeutic protein products. 2014. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM338856.pdf. Accessed 3 Sept 2014.
ICH. ICH harmonised tripartite guideline. Validation of analytical procedures: text and methodology Q2(R1). 2005. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. Accessed 16 Sep 2013.
ICH. ICH harmonized tripartite guideline. Preclinical safety evaluation of biotechnology-derived pharmaceuticals S6(R1). 2011. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S6_R1/Step4/S6_R1_Guideline.pdf. Accessed 16 Sep 2013.
ICH. ICH harmonized tripartite guideline. Quality risk management Q9. 2005. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf. Accessed 16 Sep 2013.

⟨1111⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: ACCEPTANCE CRITERIA FOR PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage, and distribution of pharmaceutical preparations.

Microbial examination of nonsterile products is performed according to the methods given in the texts on *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩. Acceptance criteria for nonsterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts and molds count (TYMC) are given in *Tables 1* and *2*. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g., direct plating methods).

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

— 10^1 cfu: maximum acceptable count = 20;
— 10^2 cfu: maximum acceptable count = 200;
— 10^3 cfu: maximum acceptable count = 2000; and so forth.

Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms

Route of Administration	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)	Specified Microorganism(s)
Nonaqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)
Rectal use	10^3	10^2	—
Oromucosal use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or1 mL)

Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms *(Continued)*

Route of Administration	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)	Specified Microorganism(s)
Gingival use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Cutaneous use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Nasal use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Auricular use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
			Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Candida albicans* (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10^2	10^1	Absence of *Staphylococcus aureus* (1 patch)
			Absence of *Pseudomonas aeruginosa* (1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulization)	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
			Absence of bile-tolerant Gram-negative bacteria (1 g or 1 mL)

Table 2. Acceptance Criteria for Microbiological Quality of Nonsterile Substances for Pharmaceutical Use

	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)
Substances for pharmaceutical use	10^3	10^2

Table 1 includes a list of specified microorganisms for which acceptance criteria are set. The list is not necessarily exhaustive, and for a given preparation it may be necessary to test for other microorganisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of microorganisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the microorganisms listed in *Table 1*, the significance of other microorganisms recovered should be evaluated in terms of the following:

- The use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract).
- The nature of the product: does the product support growth? does it have adequate antimicrobial preservation?
- The method of application.
- The intended recipient: risk may differ for neonates, infants, the debilitated.
- Use of immunosuppressive agents, corticosteroids.
- The presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

⟨1112⟩ APPLICATION OF WATER ACTIVITY DETERMINATION TO NONSTERILE PHARMACEUTICAL PRODUCTS

The determination of the water activity of nonsterile pharmaceutical dosage forms aids in the decisions relating to the following:

(a) optimizing product formulations to improve antimicrobial effectiveness of preservative systems,

(b) reducing the degradation of active pharmaceutical ingredients within product formulations susceptible to chemical hydrolysis,

(c) reducing the susceptibility of formulations (especially liquids, ointments, lotions, and creams) to microbial contamination, and

(d) providing a tool for the rationale for reducing the frequency of microbial limit testing and screening for objectionable microorganisms for product release and stability testing using methods contained in the general test chapter *Microbial Enumeration Tests* ⟨61⟩ and *Tests for Specified Microorganisms* ⟨62⟩.

Reduced water activity (a_W) will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter.

Low water activity has traditionally been used to control microbial deterioration of foodstuffs. Examples where the available moisture is reduced are dried fruit, syrups, and pickled meats and vegetables. Low water activities make these materials self-preserved. Low water activity will also prevent microbial growth within pharmaceutical drug products. Other product attributes, for example, low or high pH, absence of nutrients, presence of surfactants, and addition of antimicrobial agents, as well as low water activity, help to prevent microbial growth. However, it should be noted that more resistant microorganisms, including spore-forming *Clostridium* spp., *Bacillus* spp., *Salmonella* spp. and filamentous fungi, although they may not proliferate in a drug product with a low water activity, may persist within the product.

When formulating an aqueous oral or topical dosage form, candidate formulations should be evaluated for water activity so that the drug product may be self-preserving, if possible. For example, small changes in the concentration of sodium chloride, sucrose, alcohol, propylene glycol, or glycerin in a formulation may result in the creation of a drug product with a lower water activity that can discourage the proliferation of microorganisms in the product. This is particularly valuable with a multiple-use product that may be contaminated by the user. Packaging studies should be conducted to test product stability and to determine that the container–closure system protects the product from moisture gains that would increase the water activity during storage.

Reduced microbial limits testing may be justified through risk assessment. This reduction in testing, when justified, may entail forgoing full microbial limits testing, implementing skip-lot testing, or eliminating routine testing.

Nonaqueous liquids or dry solid dosage forms will not support spore germination or microbial growth due to their low water activity. The frequency of their microbial monitoring can be determined by a review of the historic testing database of the product and the demonstrated effectiveness of microbial contamination control of the raw materials, ingredient water, manufacturing process, formulation, and packaging system. The testing history would include microbial monitoring during product development, scale-up, process validation, and routine testing of sufficient marketed product lots (e.g., up to 20 lots) to ensure that the product has little or no potential for microbial contamination. Because the water activity requirements for different Gram-reactive bacteria, bacterial spores, yeasts, and molds are well described in the literature,[1] the appropriate microbial limit testing program for products of differing water activities can be established. For example, Gram-negative bacteria including the specific objectionable microorganisms, *Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella* species will not proliferate or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not proliferate below 0.86, and *Aspergillus niger* will not proliferate below 0.77. Furthermore, even the most osmophilic yeast and xerophilic fungi will not proliferate below 0.60, and they cannot be isolated on compendial microbiological media.[1] The water activity requirements measured at 25° for the growth of a range of representative microorganisms are presented in *Table 1*.

Table 1. Water Activities (a_W) Required to Support the Growth of Representative Microorganisms

Bacteria	Water Activity (a_W)	Molds and Yeast	Water Activity (a_W)
Pseudomonas aeruginosa	0.97	*Rhyzopus nigricans*	0.93
Bacillus cereus	0.95	*Mucor plumbeus*	0.92
Clostridium botulinum, Type A	0.95	*Rhodotorula mucilaginosa*	0.92
Escherichia coli	0.95	*Saccharomyces cerevisiae*	0.90
Clostridium perfringens	0.95	*Paecilomyces variotti*	0.84
Lactobacillus viridescens	0.95	*Penicillium chrysogenum*	0.83
Salmonella spp.	0.95	*Aspergillus fumigatus*	0.82
Enterobacter aerogenes	0.94	*Penicillium glabrum*	0.81
Bacillus subtilis	0.90	*Aspergillus flavus*	0.78
Micrococcus lysodekticus	0.93	*Aspergillus niger*	0.77
Staphylococcus aureus	0.86	*Zygosachharomyces rouxii* (osmophilic yeast)	0.62
Halobacterium halobium (halophilic bacterium)	0.75	*Xeromyces bisporus* (xerophilic fungi)	0.61

Pharmaceutical drug products with water activities well below 0.75 (e.g., direct compression tablets, powder and liquid-filled capsules, nonaqueous liquid products, ointments, and rectal suppositories) would be excellent candidates for reduced microbial limit testing for product release and stability evaluation. This is especially true when pharmaceutical products are made from ingredients of good microbial quality, when manufacturing environments do not foster microbial contamination, when there are processes that inherently reduce the microbial content, when the formulation of the drug product has antimicrobial

[1] J. A. Troller, D. T. Bernard, and V. W. Scott. Measurement of Water Activity. In: *Compendium of Methods for the Microbiological Examination of Foods.* American Public Health Association, Washington, DC, 1984 pp.124–134.

activity, and when manufacturing sites have an established testing history of low bioburden associated with their products. *Table 2* contains suggested microbial limit testing strategies for typical pharmaceutical and over-the-counter (OTC) drug products based on water activity. Other considerations, as listed above, would be applied when setting up the microbial limits testing program for individual drug products because water activity measurements cannot solely be used to justify the elimination of microbial content testing for product release.

Similar arguments could be made for the microbial limits testing of pharmaceutical ingredients. However, this would require pharmaceutical manufacturers to have a comprehensive knowledge of the pharmaceutical ingredient manufacturer's manufacturing processes, quality programs, and testing record. This could be obtained through a supplier audit program.

Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity

Products	Water Activity (a_W)	Greatest Potential Contaminants	Testing Recommended
Nasal inhalant	0.99	Gram-negative bacteria	TAMC,* TCYMC, absence of *S. aureus* and *P. aeruginosa*
Hair shampoo	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of *S. aureus* and *P. aeruginosa*
Antacid	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of *E. coli* and *Salmonella* spp.
Topical cream	0.97	Gram-positive bacteria	TAMC, TCYMC, absence of *S. aureus* and *P. aeruginosa*
Oral liquid	0.90	Gram-positive bacteria and fungi	TAMC and TCYMC
Oral suspension	0.87	Fungi	TAMC and TCYMC
Topical ointment	0.55	None	Reduced testing
Lip balm	0.36	None	Reduced testing
Vaginal and rectal suppositories	0.30	None	Reduced testing
Compressed tablets	0.36	None	Reduced testing
Liquid-filled capsule	0.30	None	Reduced testing

* TAMC = Total aerobic microbial count; TCYMC = Total combined yeast and mold count.

NOTE—The water activities cited in *Table 2* for the different dosage forms are representative, and companies are urged to test their individual products before developing a testing strategy.

Water activity, a_W, is the ratio of vapor pressure of H_2O in product (P) to vapor pressure of pure H_2O (Po) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point or indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed.

The relationship between a_W and equilibrium relative humidity (ERH) is represented by the following equations:

$$a_W = P/Po \text{ and } ERH(\%) = a_W \times 100$$

The a_W measurement may be conducted using the dew point/chilled mirror method.[2] A polished, chilled mirror is used as the condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Sample preparation should be considered as it may affect the water activity level of the material tested. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations. These instruments are typically calibrated using saturated salt solutions at 25°, as listed in *Table 3*.

Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments

Saturated Salt Solutions	ERH (%)	a_W
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753
Magnesium nitrate [$Mg(NO_3)_2$]	52.9	0.529
Magnesium chloride ($MgCl_2$)	32.8	0.328

[2] AOAC International Official Method 978.18. In: *Official Methods of Analysis of AOAC International,* 17th edition, AOAC International, Gaithersburg, Maryland.

⟨1113⟩ MICROBIAL CHARACTERIZATION, IDENTIFICATION, AND STRAIN TYPING

INTRODUCTION

Microorganisms, if detected in drug substances, excipients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished drug products, typically undergo characterization. This may include identification and strain typing, as appropriate. [NOTE—A *Glossary of Terms* is provided at the end of this chapter.] Routine characterization of microorganisms may include the determination of colony morphology, cellular morphology (rods, cocci, cell groupings, modes of sporulation, etc.), Gram reaction or other differential staining techniques, and certain key biochemical reactions (e.g., oxidase, catalase, and coagulase activity) that can be diagnostic. Microbial characterization to this level is sufficient for many risk-assessment purposes in nonsterile pharmaceutical manufacturing operations and in some sterile product manufacturing environments.

In some cases a more definitive identification of the microorganisms yields genus- and species-level identification. Beyond this, available methodologies can perform strain-level identification, which can be useful in an investigation to determine the source of the microorganism. Identification is especially common when organisms are recovered at atypically high rates or in numbers that exceed recommended levels for specific categories of products. Additionally, microbial identification is useful in aseptic processing and is necessary where sterility test positives have occurred and in the assessment of contamination recovered from failed aseptic process simulations, i.e., media fills.

Microbiological identification systems are based on different analytical methodologies, and limitations may be inherent to the method and/or arise from database limitations. Identification is accomplished by matching characteristics (genotypic and/or phenotypic) to an established standard (reference) organism such as a type strain. If a microorganism is not included in the database it will not be identified, so manufacturers should review the breadth of the database of the identification system they plan to use and its applicability to their needs. Users should consider which microbiological identification system(s) is (are) most applicable to their requirements. Bearing in mind both these limitations and the level of identification required (genus, species, strain), users also must select the appropriate technology to use in routine microbiological identification testing.

The need for microbial identification is specifically cited in USP general test chapter *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩. This chapter indicates a requirement for confirmatory identification tests for organisms that grow on selective or diagnostic media and demonstrate defined morphological characteristics. Also, USP general test chapter *Sterility Tests* ⟨71⟩ allows for invalidation of the test, if after identification of the microorganisms isolated from the test, the growth of this (or these) species may be unequivocally ascribed to faults with respect to the material and/or the technique used in conducting the sterility test procedure. USP general information chapter *Microbiological Control and Monitoring of Aseptic Processing Environments* ⟨1116⟩ recommends that microbial isolates be identified at a rate sufficient to support the environmental monitoring program.

ISOLATION OF PURE CULTURES

The first step in identification is to isolate a pure culture for analysis. This is typically accomplished by successive streaking of the colony of interest in a quadrant pattern on appropriate general microbiological solid media with the objective of obtaining discreet colonies that usually yield pure cultures. This technique also allows phenotypic expression and growth of sufficient inoculum for succeeding identification procedures. Analysts should recognize that expression of the microbial phenotype (i.e., cell size and shape, sporulation, cellular composition, antigenicity, biochemical activity, and sensitivity to antimicrobial agents) may be affected by isolate origins, media selection, and growth conditions (see *Table 1*). Therefore, the preparatory media for identification and the number of subcultures may affect the results of phenotype identification methods.

Table 1. Phenotypic Characteristics Used in Microbial Taxonomy

Categories	Characteristics
Culture	Colony morphology, colony color, shape and size, pigment production
Morphological	Cellular morphology, cell size, cell shape, flagella type, reserve material, Gram reaction, spore and acid-fast staining, mode of sporulation
Physiological	Oxygen tolerance, pH range, temperature optimum and range, salinity tolerance
Biochemical	Carbon utilization, carbohydrate oxidation or fermentation, enzyme patterns
Inhibition	Bile salt-tolerance, antibiotic susceptibility, dye tolerance
Serological	Agglutination, fluorescent antibody
Chemo-taxonomic	Fatty acid profile, microbial toxins, whole cell composition
Ecological	Origin of the organism

In contrast, the microbial genotype generally is well conserved and unaffected by cultural conditions. Therefore, once the isolation of a pure, monoclonal colony is assured, the microorganism may be analyzed without concern over the most recent growth media or the viability of the isolate. *Table 2* lists genotypic characteristics that can be determined.

Table 2. Genotypic/Phylogenetic Characteristics That Can Be Used in Microbial Taxonomy

Categories	Characteristics
Genotypic	DNA base ratio (G + C content), restriction fragment patterns, and DNA probes
Phylogenetic	DNA–DNA hybridization, and 16S and 23S rRNA sequences

Bacterial taxonomy as described in *Bergey's Manual of Systematic Bacteriology* (*Bergey's Manual*)[1] is at present accomplished by comparative analysis of genetic material. When the DNA from an unknown organism is compared to the DNA from a known organism, the degree of relatedness can be determined. Genotypic identification (*Table 2*) is accomplished through the use of DNA hybridization, restriction fragment pattern comparisons, and/or DNA probes. For example, greater than 70% relatedness with DNA–DNA hybridization indicates the organisms are the same species. Phylogenetic analysis (*Table 2*) is typically performed by comparing the base sequence of a portion of the 16S ribosomal RNA gene for bacteria, or the 23S ribosomal RNA gene for fungi. Polymerase chain reaction (PCR) is used to amplify these genes, and the amplified region is then isolated and base sequenced using an electrophoretic or dideoxy chain termination method. Comparisons can be made using validated proprietary databases or those that are publicly available. [*Caution: Publicly available databases may not be validated.*]

PRIMARY SCREENING AND CHARACTERIZATION

Microorganisms isolated on compendial media from samples of pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products may be physiologically stressed. The microorganisms will pass from a metabolic state suitable for survival under adverse ambient conditions to culture conditions that are far richer nutritionally and are at an optimal incubation temperature. This transition can be managed by careful handling of the isolates. In preparation for identification, individual representative colonies from the primary isolation media are streaked for monoclonal colonies onto solid media as described above. The first step is to determine the Gram reaction, cellular morphology, and in some cases diagnostic biochemical reactions of the bacteria isolates. This is a critical step for many phenotypic identification schemes. If the wrong characteristics are assigned to an isolate, subsequent testing may be conducted using the wrong microbial identification kit, resulting in an incorrect result. Several common preliminary screening tests are described below.

Gram Staining

Gram staining methods include the four-step method: crystal violet (primary stain), iodine (mordant), alcohol or alcohol–acetone (decolorizer), and safranin (counterstain). In the three-step method the decolorization and counterstaining steps are combined. Under optimal conditions, Gram-positive organisms retain the crystal violet stain and appear blue violet. Gram-negative organisms lose the crystal violet stain, so they contain only the counterstain safranin and appear red. Some bacteria may be Gram-variable. Common pitfalls in this method are that heat fixation may cause Gram-positive cells to stain Gram-negative, and older cultures may give Gram-variable reactions. Using too much decolorizer could result in a false Gram-negative result, and not using enough decolorizer may yield a false Gram-positive result. One variation that has advantages in some situations is to perform a methanol, rather than heat, fixation of the bacterial smear. In some cases alcohol fixation may give more consistent Gram stain results. In either method a Gram-positive and a Gram-negative control should be included to allow identification of errors in staining. Because the Gram-staining reaction must be read under a microscope, cellular morphology can be simultaneously ascertained.

Spore Staining

Spore staining can be accomplished using a malachite green stain for bacterial spores. A positive control should be included to allow identification of errors in spore staining.

Biochemical Screening

Key biochemical screening tests include (1) the oxidase test to separate Gram-negative, rod-shaped bacteria into nonfermenters (oxidase positive) and enteric (oxidase negative) bacteria, (2) the catalase test to separate *Staphylococci* (catalase positive) from *Streptococci* (catalase negative), and (3) the coagulase test to separate *Staphylococci* into coagulase negative (presumptively nonpathogenic) and coagulase positive (more likely pathogenic) *Staphylococci*.

For many types of investigations and routine surveying of manufacturing environmental bioburden, these few tests can provide sufficient information for ongoing evaluation. However, when circumstances dictate greater in-depth assessment, identification to the genus, species, or strain level can yield valuable insights about the nature and source of environmental biobur-

[1] *Bergey's Manual of Systematic Bacteriology, 2nd Edition,* 2003.

den. Also, microbial identification to the species and even strain level can be critical in assessing and mitigating risk from microbial contamination.

MICROBIAL IDENTIFICATION BY PHENOTYPIC METHODS

Phenotypic methods use expressed gene products to distinguish among different microorganisms. Generally, these require a relatively large number of cells in pure, monoclonal culture. Recovery and growth methods for microbial enumeration and identification are limited by the length of incubation and the fact that many organisms present in the environment are not recovered by general microbiological growth media. Additionally, freshly isolated, stressed microorganisms by subculture from primary recovery may not result in a full expression of phenotypic properties. However, methods based on carbon utilization and biochemical reaction, as well as fatty acid profiles by gas–liquid chromatography and whole-cell composition by MALDI-TOF mass spectrometry, are always based on inocula development for a specific identification system. These systems rely on specified culture media and incubation conditions to achieve consistent identification. Phenotypic microbial identification methods are successfully used in food, water, clinical, and pharmaceutical microbiological testing laboratories.[2] Phenotypic microbial identification methods provide information that enables microbiologists to make informed decisions regarding product risk and to recognize changes in environmental microflora. In many quality control investigations, phenotypic identification alone is sufficient and will enable scientists to conduct a thorough investigation and to recommend appropriate corrective actions as needed.

MICROBIAL IDENTIFICATION BY GENOTYPIC METHODS

Genotypic microbial identification methods are theoretically more reliable because nucleic acid sequences are highly conserved in most microbial species. Applicable genotypic methods include DNA–DNA hybridization, PCR, 16S and 23S rRNA sequencing, multilocus sequence typing (MLST), pyrosequencing, DNA probes, and analytical ribotyping. These methods can be technically challenging for microbiologists. They also require more expensive analytical equipment and supplies. Often these analyses are conducted by contract laboratories, government laboratories, universities, research institutes, or specialized laboratories within industrial firms. Therefore, the use of genotypic identification methods is typically limited to critical microbiological investigations such as product failure investigations. Further, if strain-level identification is done in the course of an investigation, analysts must ensure that the method is appropriate.

DNA sequencing of the first 500 base pairs of the 16S rRNA sequence is useful for identification to the species level but may not provide sufficient power to resolve among closely related species or strains of the same species. In contrast, Southern hybridization of restriction endonuclease digests is powerful and can be effective in demonstrating differences between two strains. If the banding patterns appear identical, this shows only that restriction endonuclease has similar cleavage sites in that region of the two organisms. Demonstration that the two organisms are the same should include two or more different restriction endonuclease digests, each of which yields bands in the area of interest. All bands from the two organisms must be identical.

In contrast to microbial identification, nucleic acid-based methods can be used to screen for specific microorganisms. The steps associated with this activity are sample collection, nucleic acid extraction, target amplification, hybridization, and detection. The problem of amplifying DNA from nonviable bacterial cells can be overcome by using reverse transcription to convert rRNA that is transitional, hence related to viability, to DNA for PCR amplification. Issues include the detection of microbial variants, limits of detection, matrix effects, positive cutoff verification, instrument and system carry-over, diagnostic accuracy, and reproducibility.

VERIFICATION OF MICROBIAL IDENTIFICATION METHODS

Microbial identification tests include serological tests, chemical reagents, reference organisms, and instrumentation. The verification of an identification test system can include one of the following: (1) using an existing system for parallel testing of microbial isolates obtained from routine testing (the number of isolates tested may be as high as 50, and any discrepancies in identification can be arbitrated using a referee method); (2) testing 12–15 known representative stock cultures of different commonly isolated species for a total of 50 tests; or (3) confirming that 20–50 organism identifications, including 15–20 different species, agree with the results of a reference laboratory testing of split sample.[3] In each case the appropriate quality control organisms, as recommended by the supplier and the compendia, should be included in the verification process.

With identification systems, verification of the identity of the species should be evaluated and the level of agreement should be considered. Typically greater than 90% agreement can be achieved with samples of microorganisms that are appropriate for the identification system. Groups of organisms that are challenging to identify (e.g., nonfermenting bacteria, corynebacte-

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[2] O'Hara, C.M., M.P. Weinstein, and J.M. Miller. Manual and automated systems for detection and identification of microorganisms. *ASM Manual of Clinical Microbiology, 8th Edition,* 2003.

[3] Cumitech 31. *Verification and Validation of Procedures in the Clinical Microbiology Laboratory.* Elder, B.L., S.A. Hansen, J.A. Kellogg, F.J. Marsik, and R.J. Zabransky, ASM, February 1997.

ria, and coagulase-negative *Staphylococci*) may be included, when appropriate, in the verification process but may yield lower levels of agreement.

The hierarchy of microbial identification errors in descending order of impact is (1) misidentification to genera, (2) misidentification to species, and (3) no identification. Misidentification could lead to inappropriate corrective and preventive actions and product disposition.

A microbial identification system may not be able to identify an isolate because the organism is not included in the database, the system parameters are not sufficiently comprehensive to identify the organism, the isolate may be nonreactive in the system, or the species may not have been taxonomically described. Such isolates can be sent to the supplier of the microbial identification system for additional study and, if appropriate, added to the database. Alternatively, genotypic identification tests can be conducted, and the species can be added to an in-house database. Misidentification is more difficult to determine, but any microbial identification should be reviewed for reasonableness in terms of the microorganism's morphology, physiological requirements, and source of isolation. Organisms identified only to genus may be common for the numerous nonpathogenic species of *Staphylococcus, Corynebacterium* (and other small pleomorphic Gram-positive rods), and *Micrococcus*.

The most important verification tests are accuracy and reproducibility. These measurements can be defined as follows:

Accuracy % = (Number of correct results/Total number of results) × 100

Reproducibility % = (Number of correct results in agreement/Total number of results) × 100

The user should establish suitable acceptance criteria for accuracy and reproducibility, taking into account method capability.

Other measurements are sensitivity, specificity, and positive and negative predictive value. These measurements are best illustrated by an example. A clinical microbiology laboratory compared the frequency of isolation of a DNA hybridization probe to a culture method for the sexually transmitted bacterium *Neisseria gonorrhaeae*.[3] The frequency of isolation from clinical specimens was historically 10%. The lab ran 100 split samples, and the results are presented in *Table 3*.

Table 3. Comparison of the Distribution of Negative and Positive Results for the DNA Probe and Culture Methods

	Culture Results	
DNA Probe Results	Positive	Negative
Positive	9	2
Negative	1	88

Sensitivity = [9/(9 + 1)] × 100 = 90%

Specificity = [88/(88 + 2)] × 100 = 97.7%

Positive Predictive Value = [9/(9 + 2)] × 100 = 81.8%

Negative Predictive Value = [88/(88 + 1)] × 100 = 98.9%

Note that the positive predictive value (PPV) is not intrinsic to the test; it also depends on the prevalence of the microorganism in clinical samples. PPV is directly proportional to the prevalence of the disease or condition. In this example, if the group of people tested had included a higher proportion of people with infection, then the PPV would probably be higher and the negative predictive value (NPV) lower. If all persons in the group had infection, the PPV would be 100% and the NPV 0%. The mathematical derivation of these functions is outlined in *Table 4*.

Table 4. A Two-Row by Two-Column Contingency Table with Respect to the Reference Culture Method and the Alternate PCR Method (After ISO 5725-1 and 5725-2 2004)*

	PCR		
Culture	**Positive**	**Negative**	**Sum**
Positive	a True Positive	b False Negative	a + b
Negative	c False Positive	d True Negative	c + d
Sum	a + c	b + d	

* ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions and ISO 5725-2:1994 Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic methods for the determination of repeatability and reproducibility of standard measurement methods.

Inclusivity (%) = [a/(a + b)] × 100

Exclusivity (%) = [d/(c + d)] × 100

Positive Predictivity (%) = [a/(a + c)] × 100

Negative Predictivity (%) = [d/(b + d)] × 100

Analytical Accuracy (%) = [(a + d)/(a + b + c + d)] × 100

Kappa Index = 2(ad – bc)/[(a + c) × (c + d) + (a + b) × (b + d)]

General Chapters

Phylogenetic Considerations

The second edition of *Bergey's Manual* represented a major departure from the first edition, and also from the eighth and ninth editions of the *Manual of Determinative Bacteriology*. The organization of content in *Bergey's Manual* follows a phylogenetic framework, based on analysis of the nucleotide sequence of the ribosomal small subunit 16S RNA, rather than a phenotypic structure.

Phylogenetic trees or dendrograms show the closest genetically related organisms. The application of this technology has resulted in taxonomic revisions and the renaming of some well-known microorganisms; e.g., the fungus *A. niger* ATCC 16404 was renamed *A. brasiliensis*. In general, organisms with relatedness less than or equal to 97% are considered different genera and those with relatedness less than or equal to 99% are considered different species,[4] but there are many exceptions to this generalization.

Differences in genotype and phenotype are relatively uncommon, e.g., same or very similar genotype shared by microorganisms with different phenotypes, similar phenotypes but different genotypes, and microorganisms that are genotypically too distant to be the same species or genus. The concept of polyphasic taxonomy[5] that refers to assembly and use of many levels of information, e.g., microbial characterization, phenotypic and genotypic data, and origin of the microorganisms, can be successfully applied to microbial identification. This avoids decisions made solely using genotypic data that make no sense when the microbial characteristics, testing history, and source of isolation are considered.

GLOSSARY

Microbial classification: The arrangement of microorganisms into taxonomic groups based on their similarities and relationships.

Microbial identification: The determination of which broad group (e.g., bacteria, yeast, or mold) or narrow group (e.g., genus and/or species) to which a laboratory isolate belongs.

Microbial characterization: The use of colony growth, cellular morphology, differential staining, and key diagnostic features to characterize a laboratory isolate for trending and investigative purposes without identification, e.g,. nonpathogenic Staphylococci.

Mol %GC: The molecular percentage of guanine–cytosine range within the chromosomal DNA. [NOTE—The %GC + %AT = 100%.]

Phylogenetic species: A species consisting of many strains including the type of strain that shares at least 70% total genome DNA–DNA hybridization and less than 5° ΔTm (difference in melting point of the hybrid).

Polyphasic taxonomy: Taxonomy that assembles and assimilates many levels of information from molecular, physiological, morphological, serological, or ecological sources to classify a microorganism.

Relatedness: The extent of relationship or similarity of two (or more) organisms on a Phylogenetic Tree or a Dendrogram.

rRNA Sequence: The DNA sequences that encode rRNA used in protein synthesis are highly conserved among microorganisms of a common ancestry. They are used to determine the phylogenetic distance between organisms and are useful in microbial taxonomy and identification.

Strain: A specific isolate of a species that is maintained in pure culture and is characterized. The type strain is representative of the species that provides a reference for the species based on its historic isolation, characterization, and deposition in recognized culture collections.

Strain typing: Strain typing is an integral part of epidemiological investigations in clinical and public health microbiology. Methods including pulsed-field gel electrophoresis, riboprinting, arbitrarily primed polymerized chain reaction, and whole genome ordered restriction or optical mapping can be used to demonstrate that microbial species are the same strain and most likely are from a common source.

〈1115〉 BIOBURDEN CONTROL OF NONSTERILE DRUG SUBSTANCES AND PRODUCTS

INTRODUCTION

In terms of microbiological contamination risk control, there are two broad categories of drug products: (a) sterile products, in which the bioburden is essentially eliminated using validated methodologies, and (b) nonsterile products for which the final

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[4] J.E. Clarridge III. The Impact of 16S rRNA Gene Sequencing Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases, *Clin. Microbiol. Rev. 17 (2)* 840–862, 2004.

[5] Gillis, M., P. Vandamme, P. De Vos, J. Swings and K. Kersters. Polyphasic Taxonomy. *Bergey's Manual of Systematic Bacteriology, 2nd Edition*, 2003.

General Chapters

product bioburden is controlled to appropriate levels based on product attributes, route of administration, and target patient population.

Microbial content in nonsterile products is controlled to a level consistent with patient safety. Use of excessive controls that would add complexity or cost without a commensurate safety benefit is not advantageous in terms of added value to either the patient or the manufacturer. Therefore, a pragmatic scientific approach to management of the microbial bioburden in nonsterile products requires consideration of patient risk and contamination control objectives to achieve a practical and appropriate level of risk management.

A critical consideration in ensuring product quality is to prevent conditions within the manufacturing facility or manufacturing process that favor the proliferation or ingress of microorganisms. Microbial growth in excipients, components, and drug substances is a concern because it creates the possibility that viable microbial content could reach unacceptable levels. Bioburden levels in the ranges of those recommended in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ are recognized as safe and do not pose risk of infection or microbial toxins. Manufacturers should have a clear understanding of situations that could favor microbial growth within their facilities and materials and should implement practical countermeasures.

This chapter outlines a risk-based approach to the control of contamination in nonsterile product manufacturing. The manufacture of nonsterile products and the management of their microbiological content are distinctly different from those required for sterile products. Sterile products are administered parenterally by means of injection or are applied topically to sensitive tissues where the risk of infection is comparatively high.

In contrast, nonsterile products are administered to regions of the human body that are rich in microbial flora and have physical or immunological barriers to infection. Examples include the oral cavity, skin, nasopharynx, vagina, and rectum, which harbor a high and diverse viable microbial population. Recent findings from the human microbiome project (*1*) underscore the enormous size and diversity of bacterial populations associated with humans. A healthy adult has a bacterial population that averages approximately 10^{14} bacteria, a number that exceeds the individual's own cells by a factor of 10. All humans carry on or in their bodies microorganisms that under certain conditions may cause infection in other humans. Because these microorganisms are present on humans means they may be present transiently in the nonsterile manufacturing environment. Their ubiquity in nature and infrequent association with infection confirms that the risk associated with them is very low. However, good hygienic controls and selection of suitable gowning systems are important considerations for products that are intended for patients who may be immunocompromised.

The following list provides a hierarchy of broad categories of nonsterile pharmaceutical products with respect to potential risk of microbiological contamination (from high to low) (*2*):

- metered-dose and dry powder inhalants
- nasal sprays
- otics
- vaginal suppositories
- topicals
- rectal suppositories
- oral liquids (aqueous)
- liquid-filled capsules
- oral tablets and powder-filled capsules

When formulators evaluate the susceptibility of nonsterile pharmaceutical products to microbial hazards, considerations include whether the active ingredient has inherent antimicrobial activity, the microbiological content of excipients, inclusion of antimicrobial preservatives in the formulation, and water activity. In addition, manufacturers should consider whether processing steps and hold periods could result in changes in the bioburden.

Nonsterile products are expected to have some bioburden, which should be controlled within a suitable range (see ⟨1111⟩). The risk of infection resulting from a nonsterile drug product generally is low, regardless of the route of administration, provided appropriate precautions and procedures are followed. General chapters *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩ and *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩ provide methods, and ⟨1111⟩ provides information for the evaluation of microorganisms isolated during nonsterile drug product manufacturing. It is not possible to provide a comprehensive product-by-product list of objectionable microorganisms. The degree to which any organism may be objectionable depends on the product attributes, route of administration, and patient population. Manufacturers are responsible for determining whether microorganisms recovered from drug products are objectionable. In general, objectionable microorganisms are those that are known to be truly pathogenic, considering the product's route of administration. Guidance for risk factors to be considered is also provided as a bulleted list in ⟨1111⟩. Risk may also arise where microorganisms are able to proliferate in sufficient numbers that could result in an unacceptable level of patient risk to a level above the ranges recommended in ⟨1111⟩. Microbiological risk should be assessed on a case-by-case basis during the development of a new product and should be evaluated during the validation of the manufacturing process.

The proliferation of microbial contamination in a production facility, in products, or in product ingredients is an objectionable condition. Microbial proliferation within a facility creates conditions that favor the spread of contaminants, potentially in dangerous numbers, into ingredients, primary packaging materials, and even into product itself. Proliferation of microorganisms in ingredients or production intermediates must be prevented because it creates a risk due to microbial toxin and could result in damage to the chemical and pharmacological properties of the drug.

U.S. REGULATORY GUIDANCE DOCUMENTS

The U.S. Code of Federal Regulations includes the Food and Drug Administration's Good Manufacturing Practice (GMP) requirements, which are found in Part 211 (*3*). These regulations contain general requirements for the manufacture of pharmaceutical products. Pertinent sections of 21 CFR 211 include: *211.42 Design and construction*—requires that buildings used for pharmaceutical manufacturing and associated activities are of suitable size, construction, and location; *211.46 Ventilation, air filtration, air heating and cooling*—requires adequate equipment to control microorganisms, dust, humidity, and temperature and differential air pressures be provided when appropriate for drug product manufacturing; *211.56 Sanitation*—requires written procedures for use of suitable rodenticides, insecticides, fungicides, fumigating agents, and cleaning and sanitizing agents, and written procedures shall be designed to prevent the contamination of equipment, components, drug product containers, closures, packaging, and labeling materials; *211.82 Receipt and storage of untested components, drug product containers, and closures*—requires an acceptance, quarantine, testing, and release procedure for components and containers; *211.110 Sampling and testing of in-process materials and drug products*—requires testing of in-process manufacturing controls that will include bioburden determination. Objectionable organism considerations: *21 CFR 211.84(d)(6)*—requires microbiological testing of incoming containers and components with potential for microbiological contamination that is objectionable in view of their intended use; *21 CFR 211.113(a)*—requires written procedures directed to control of objectionable microorganisms in drug products not required to be sterile; *21 CFR 211.165(b)*— requires appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms.

MICROBIAL CONTROL CONSIDERATIONS DURING PRODUCT DEVELOPMENT

A formal risk assessment program that identifies risk modalities and assigns critical control points for manufacture of nonsterile product is useful. Hazard Analysis and Critical Control Point programs (*4*) are widely used to assess and mitigate microbial risk in food manufacturing and may also be useful for manufacturers of nonsterile drug products. Points to be considered by pharmaceutical microbiologists when they assess the potential risk associated with nonsterile drug product manufacture are listed below:

- synthesis, isolation, and final purification of the drug substance
- microbiological attributes of the drug substance
- microbiological attributes of excipients and intermediates
- formulation and chemical and physical attributes of the drug product
- antimicrobial properties of the material, e.g., water activity or others
- manufacturing process
- delivery system
- packaging
- storage conditions for intermediates and the finished dosage form
- route of administration
- expected treatment procedure and dosage regimen
- population to which the product is delivered (e.g., neonates, immunocompromised patients, etc.)

Thorough consideration of these factors is valuable in defining appropriate manufacturing facility requirements and process control point measures that limit conditions that favor microbial proliferation and/or ingress.

MICROBIAL CONTROL CONSIDERATIONS DURING MANUFACTURING

Although many factors (*Figure 1*) can result in the introduction of microorganisms, some of these are more likely to result in microbial contamination. These manufacturing factors are, in descending order (*5*): (1) ingredient water, (2) pharmaceutical ingredients, (3) process equipment, (4) manufacturing personnel, and (5) manufacturing environment.

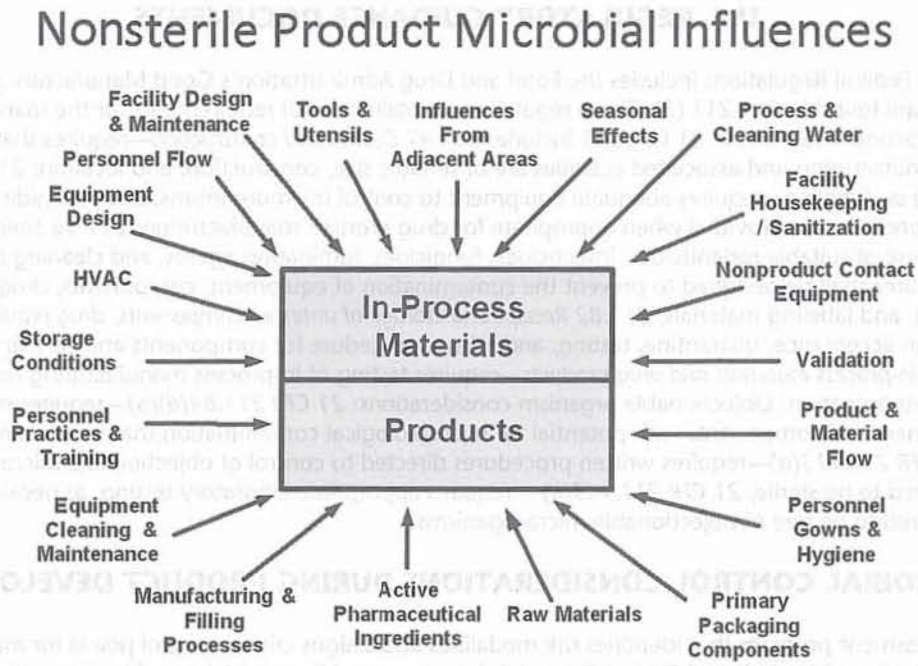

Figure 1. Factors that contribute to nonsterile product bioburden.

Water Systems and Use

Water used in active ingredient manufacturing, formulation, cleaning, and housekeeping is the single most important risk element contributing to the contamination of nonsterile products. The quality or type of water used for nonsterile product formulation and final rinse of clean equipment should be chosen based on product risk. Purified waters used in pharmaceutical manufacturing are deionized and thus do not contain chlorine to control microbial growth. Substantial populations of Gram-negative rod-shaped bacteria and some molds are able to grow in such purified dechlorinated water, particularly in holding tanks at or around ambient temperatures. Standing water should be drained or physically removed quickly and efficiently from production vessels and equipment, as well as work surfaces and floors. Chlorinated potable water (city water) may be appropriate for some cleaning, housekeeping, and sanitization activities. Water quality for processing application uses should be determined on a case-by-case basis. Additional guidance for water system design and operation can be found in *Water for Pharmaceutical Purposes* 〈1231〉.

Process waters used for manufacturing of excipients, and, in some cases, active ingredients for nonsterile products present a substantial risk for microbial colonization and proliferation, particularly for ingredients of natural origin that have received minimal processing to reduce bioburden or to control microbial proliferation. Formulating and manufacturing equipment can be a source of contamination, and risks are higher when water and ingredients that are susceptible to microbial survival or growth are used. Therefore, cleaning, drying, and, where appropriate, sanitization of manufacturing equipment can be beneficial, but disinfectant residues should be limited in the operating environment and should be removed from product-contact surfaces. The isolation of waterborne organisms, particularly Gram-negative rods, is a likely indicator of failure to remove standing water on equipment and environmental surfaces.

Active Pharmaceutical Ingredients, In-Process Materials, and Excipients

Ingredients and excipients used in nonsterile product manufacturing processes are important sources of microbiological contamination. Vendor evaluation, specifications, testing, package selection, shipping, storage conditions, expiry dates, and assessment for likely contamination or proliferation risk are all critically important in the reduction of microbial risk associated with these materials. Of particular concern are unprocessed materials of natural origin, those that have high water activity (see *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* 〈1112〉 for additional information), synthetic processes with aqueous isolation steps or open processing, and biological processes with limited downstream purification or lacking defined microbial removal step(s). In-process bioburden monitoring points should be established at points in the process that are either immediately before or immediately follow a potential bioburden reduction process (such as an organic solvent extraction, heating, large change in pH) and/or immediately prior to final fill as determined by the risk analysis performed as suggested in the section *Microbial Control Considerations during Product Development*.

Manufacturers should sample incoming materials and should ensure proper contamination-control conditions for weighing and material addition to process equipment. All sampling and weighing equipment should be properly cleaned, sanitized,

stored, and identified. The activities and the associated controls implemented to prevent microbial colonization and proliferation should be based on a documented, prospective risk-assessment and contamination-control strategy.

Pharmaceutical ingredients of consistently suitable microbial quality are an important element of the microbiological control program for nonsterile products. Procurement of ingredients of appropriate microbial quality requires the identification of vendors with the demonstrated capacity to produce drug substances or excipients of suitable quality. Supplier survey assessments may be conducted periodically to establish that the supplier has a well-designed and maintained microbiological control program for its manufacturing and primary packaging facilities. Materials that have low water activity, possess high or low pH, are not of natural origin, are inherently antimicrobial, or contain an antimicrobial preservative are generally not at risk for microbial proliferation. Risk assessments should consider ingredient characteristics regarding microbial survival, support of microbial growth, or frank antagonism to microbial survival.

The introduction of moisture into stored materials notably increases the risk of microbial contamination. Condensation in storage tank headspace or impermeable storage containers can result in contamination of materials with waterborne organisms or fungi even when the product under storage is expected to preclude microbial colonization or proliferation.

Microbiological examination of pharmaceutical ingredients (see chapters ⟨61⟩, ⟨62⟩, and ⟨1111⟩) can provide important insights into the microbiological quality of drug substances and excipients.

Primary packaging and intermediate containers (e.g., drum liners, plastic bags, and so on) can be a source of microbial contamination, and manufacturers should consider their initial quality, storage conditions, preparation, and handling procedures.

MICROBIAL CONTROL OF DRUG SUBSTANCE MANUFACTURING

The approaches to microbial control described in this chapter can be applied with minimal change to drug substances manufactured either by biological processes or organic synthesis. The processes in organic synthesis often use extremes of temperature, pressure, pH, and other conditions that inhibit or actively destroy microorganisms. When the chemical process for manufacturing a drug substance is complex, the final steps often have the greatest potential effect on the bioburden. As is the case for drug product manufacturing, the water used in the process presents the greatest challenge to microbial control. The types of water used in these processes vary. Potable water typically is used in early-stage processing and cleaning, and Purified Water and Water for Injection often are used for downstream processing and cleaning. Use of Water for Injection is typically restricted to the final steps of bulk chemical or biological drug substance manufacturing that will ultimately be used in the formulation of sterile products. These processes are frequently performed almost entirely within closed vessels, which minimizes external facility considerations. Some process equipment is suitable for clean-in-place operations that use agents that are not only effective cleansers but also in some cases inhibit microbial growth/proliferation. The materials used in drug substance manufacturing include natural substances, organic compounds of various types, inorganic salts, acids, bases, and organic solvents, and the potential for microbial contamination from these materials clearly is variable. Personnel interventions in these processes are limited by the nature of the equipment and consideration for worker safety, and in biologics processing and certain late stages of organic synthesis the manufacturing environments may be classified (e.g., ISO 7 and ISO 8). Limiting the involvement of personnel and use of controlled environments both reduce opportunities for microbiological contamination.

Equipment Design and Use

When possible, specifications for the selection of equipment that will be used in the manufacture of nonsterile products should include sanitary design. Equipment and utensils should be cleanable so contaminants and residual products can be reliably removed. Equipment should use sanitary fittings and should be designed for easy use of cleaning and sanitizing agents and complete rinse water drainage. Residual water in tanks, piping, or on equipment surfaces introduces the risk of colonization by waterborne organisms. Manufacturing equipment that cannot be cleaned in place should be readily accessible for manual cleaning, and parts that must be cleaned out of place should be not only easily accessible but also readily or easily removable. A further consideration is the compatibility of equipment with the typical range of disinfectants, including sporicides, used in cleaning procedures to sanitize equipment.

The preferred material of construction for equipment and utensils that will be in contact with product is austenitic stainless steel. Manufacturers should consider the surface finish on product-contact materials, for which a roughness average (RA) of 15–20 μm is a reasonable rule of thumb. Surfaces for general equipment and machine surfaces need not be polished beyond these RA values. Other materials of construction should be nonporous, smooth, and compatible with the products and cleaning materials. Process piping systems also should follow sanitary design principles and should be sloped to facilitate drainage, and the equipment should contain flush gaskets to prevent material build-up and to facilitate cleaning. The 3A Sanitary Standards (see www.3-A.org) provide generally useful guidance for process layout and design and machinery selection. Equipment cleaning procedures should be detailed and should ensure that the equipment is completely dry after cleaning and is stored in a manner that prevents microbial proliferation. Manufacturers should implement procedures for the protection of cleaned equipment and utensils before their next use. Cleaning and sanitization process validation should include the evaluation of microbial content both after sanitization and before use. Properly designed storage protection should mitigate the possibility of microbial growth before use, so after proper storage conditions are validated ongoing monitoring of equipment and utensils should not be required. Surface microbial sampling either immediately after cleaning or immediately before use must be done with caution; media residues and residual moisture must be carefully eliminated if sampling is performed.

Manufacturers should evaluate whether products that are manufactured using a piece of processing equipment may, under some processing circumstances, promote the growth of microorganisms. This evaluation is necessary to properly establish processing hold times and to define equipment use conditions following cleaning. The use of sanitizing agents on product-contact surfaces is not required when the cleaning procedures that remove chemical residues also remove microorganisms. In addition, there is a risk of product contamination with sanitization agents. With a validated cleaning process, the absence of chemical residues and visible standing water may provide assurance of the cleanliness of the equipment without routine chemical disinfection and also may obviate the need for microbiological monitoring of the equipment.

Personnel

In addition to maintaining personal hygiene, operators should be trained and dressed appropriately for the function they are performing (*Table 1*).

Table 1. Gowning in Manufacturing Areas

Protective Clothing	Operators in Formulation and Primary Packaging Areas
Plant uniform or plant uniform with overall for high-risk product and environment	Yes
Hair/beard covering	Yes
Safety glasses	Yes
Dedicated shoes or shoe coverings	Yes
Gloves	Yes (if in direct product contact)
Face masks	Yes (if in direct product contact)

The Manufacturing Environment

As noted, the environmental risks and controls for nonsterile products are different from those for aseptically manufactured sterile products. Unlike aseptic processing for which facility requirements are generally uniform in specification and performance, nonsterile product manufacturing environments typically involve diverse products and microbial contamination control requirements. In general, liquid, cream, or ointment products require a greater level of contamination risk mitigation than do solid dosage forms.

Common design elements to control microbial contamination may include the following:

- Walls, ceilings, and floors are constructed of nonporous materials that are readily cleanable and are resistant to cleaning agents and disinfectants.
- Floor drains are permitted in nonsterile product manufacturing areas provided that they can be closed during processing or fitted with a suitable air break if they are open during area and equipment cleaning.
- Access should be limited to essential personnel.
- Material, equipment, and personnel flows should avoid contamination.
- Ventilation and air filtration should be adequate to maintain the specified cleanliness, space pressurization (if required), temperature, and relative humidity.
- Good housekeeping and good general hygiene should be applied at all times.
- Cleaning and use status of all tools and implements used in production and all process equipment should be known at all times.
- Product-contact or water-supply tubing, valves, and fittings should be cleaned and sanitized according to a defined schedule, should be stored dry, and should be labeled with respect to status.
- Manufacturers should implement a formal housekeeping and sanitization program for operating areas, corridors, equipment storage, material staging, and other common areas.

Classified environments are not required for nonsterile product manufacturing, e.g., those specified in ISO 14644-1 (*6*). ISPE Baseline Guide No. 2, Oral Solid Dosage Forms (*7*) provides minimal acceptable design features for facilities for manufacturing nonsterile products.

MICROBIAL ASSESSMENT OF NONSTERILE PRODUCT MANUFACTURING ENVIRONMENTS

As described above, microbiological monitoring of the manufacturing environment can serve as an adjunct to control and generally is a qualitative assessment tool in a properly implemented formal risk-based microbiological control program. A monitoring program commensurate with the product bioburden risk can help confirm the effectiveness of microbiological controls and may facilitate early detection of potential problems. Microbial methods and practices for aseptic facilities may be used, but the contamination recovery rates defined in *Microbiological Control and Monitoring of Aseptic Processing Environments* 〈1116〉 are not intended for nonsterile environments. Classified environments of the same class do not necessarily have similar microbiological control capabilities. Because the majority of organisms recovered in environmental monitoring are of human origin, the levels of transient contamination recovered depend largely on the level of human activity and gowning requirements. Gown-

ing requirements are not as extensive in nonsterile product manufacturing as they are in aseptic processing, and thus in nonsterile environments manufacturers can monitor bioburden with reduced frequency and with expectations of higher bioburden recovery. Periodic assessments of production plant hygiene can offer useful insights into the effectiveness of the facility's cleaning and environmental controls.

During nonsterile product manufacturing, microbiological monitoring of the environment need not be as rigorous as that required during aseptic processing. Similarly, during nonsterile product manufacturing microbial risk mitigation is different from that for sterile products. In nonsterile products, manufacturers can expect intrinsic microbial bioburden that, properly controlled, does not result in risk to the end user. Manufacturers should establish acceptable levels of microorganisms within each product (*3*) and should sufficiently identify microorganisms to gain an appropriate understanding of bioburden patterns and seasonal variability.

Microbial Sampling

During nonsterile product manufacturing, the air sampling methods used for environmental monitoring are active or passive. Active devices sample appropriate air volumes and deposit viable organisms on solid media plates or strips. Results typically are expressed as colony-forming units per unit volume although alternate methods may be used in lieu of recovery and growth on media. Passive sampling in the form of settling plates can be used in lieu of active air samplers.

Personnel monitoring typically is not required in nonsterile product manufacturing except when near-aseptic gowning materials are employed.

Sampling locations should be selected based on risk evaluation followed by a general hygienic survey of the environment. High-traffic areas where materials are transferred into and out of the area may be particularly prone to transient microbial contamination. Microbiological monitoring is not required in areas beyond the point where product has been placed into primary packaging containers.

Alternate nongrowth-based microbiological methods can be substituted for growth-based methods at the option of the user. Because there are no standardized environmental sampling methods and because monitoring is intended as a qualitative evaluation of general facility hygiene, there is no need for comparative studies between growth- and nongrowth-based methods. A small number of parallel samples is sufficient to establish a comparative baseline for an environment.

The frequency of monitoring should reflect the potential risk associated with the dosage form (see *Introduction*). Additionally, some products may have innate antimicrobial activity because of their attributes such as low water activity or inclusion of an antimicrobial preservative or an active ingredient that is itself an antimicrobial agent, e.g., an antibiotic or antitumor agent. Products that are resistant to microbial colonization or have microbiocidal or microbiostatic characteristics require little or no microbiological monitoring.

In general, environments for tablet and powder- and liquid-filled capsule manufacturing should require no monitoring or infrequent monitoring. Monitoring programs should be risk based, and the frequency and number of sampling sites should reflect the risk level. Manufacturing areas for higher-risk dosage forms such as inhalant products require more frequent monitoring and typically are manufactured in rooms classified to a particulate air quality level, e.g., ISO 8.

For most nonsterile product manufacturing environments, because of their limited environmental controls and comparatively low product risk, the establishment of alert and action levels may not be required. Environmental monitoring is considered an informational survey of the general hygienic conditions of the environment and should not be used in product-release decisions. Monitoring of unclassified environments is not required.

General information chapter *Microbial Characterization, Identification, and Strain Typing* 〈1113〉 contains general information about microbial characterization. In most nonsterile hygienic assessments, characterization of the microorganisms, cellular morphology, Gram reaction, and simple diagnostic testing are sufficient.

Active Measures for Microbial Control

In addition to facility and process design considerations and equipment cleaning and storage controls, there are instances in which active means for addressing the contamination risk are required. The microbiological control of nonsterile products can be enhanced by the adoption of direct contamination control processes such as the following:

- decontamination of product contact surfaces, materials, and containers, typically by means of heat treatment (in the most critical applications sterilization can be used)
- chemical or physical (e.g., dry or moist heat) bioburden reduction treatments for raw materials and active ingredients
- use of closed, cleaned or decontaminated systems for handling and transfer of materials
- use of disposable components or utensils
- improved gowning materials for operational personnel
- use of classified environments in high-risk operations

These measures can be applied as required to improve the microbial contamination control during the manufacture of nonsterile products.

OVERALL MANAGEMENT OF A MICROBIOLOGICAL CONTROL PROGRAM

The management of a successful microbiological control program includes the following: identification of suitable suppliers of pharmaceutical ingredients and excipients that have good microbiological quality; conducting a microbial risk assessment of the manufacturing process and packaging system; and the establishment of an appropriate monitoring and control system.

Although environmental contamination is by no means the most significant cause of nonsterile product recalls or contamination events, environmental monitoring may be a program adjunct to the microbiological control program. Microbial monitoring is an assessment and is not by itself a contamination control activity. There have been no scientifically controlled studies demonstrating what linkage, if any, exists between airborne or surface monitoring results and microbiological safety of the final product.

The microbiological contamination control program should be developed for identifying and controlling product risk, based on a formal assessment of risk modalities. The risk analysis should result in the identification of critical control points and should facilitate proper equipment selection, process layout and design, and facility design requirements.

Critical factors for the prevention of microbiological contamination during nonsterile product manufacturing are the control of the microbiological quality of ingredients and water, along with the development of proper cleaning and sanitization procedures. Microbiological monitoring does not mitigate risk, but it may serve as a sentinel.

No monitoring program can provide the assurance of contamination control as effectively as sound, proactive and preventive measures. Consistent control of contamination can be achieved mainly by an overall process evaluation assessing each of the control elements described above via risk assessment. Risk assessment may be coupled with active evaluation studies to ensure that appropriate measures are in place to prevent conditions conducive to contamination.

REFERENCES

1. Faust K, Sathirapongsasuti JF, Izard J, et al. (2012) Microbial co-occurrence relationships in the human microbiome. *PLoS Comput Biol.* 2012;8(7):e1002606.
2. Cundell AM. Risk based approach to pharmaceutical microbiology. In: Miller MJ, ed. *Encyclopedia of Rapid Microbiology Methods.* River Grove, IL: Davis Healthcare International Publishing: 2005.
3. 21 *CFR* 211. Good Manufacturing Practice for Finished Pharmaceuticals.
4. FDA. Hazard analysis and control point principles and application guidelines. 1997. http://www.fda.gov/Food/GuidanceRegulation/HACCP/ucm2006801.htm. Accessed 07 February 2013.
5. Anderson AS, Bassett G, Burke MT, et al. Microbiological monitoring of environmental conditions for nonsterile pharmaceutical manufacturing. *Pharm Technol.* 1997;21(3):58–74. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q9/Step4/Q9_Guideline.pdf. Accessed 07 February 2013.
6. ISO. 14644-1:1999. Cleanrooms and associated controlled environments—part I: classification of air cleanliness. 1999. http://www.iso.org/iso/catalogue_detail.htm?csnumber=25052. Accessed 07 February 2013.
7. ISPE. Baseline guide volume 2: oral solid dosage forms. 2009. http://www.ispe.org/baseline-guides/oral-solid-dosage. Accessed 07 February 2013.

⟨1116⟩ MICROBIOLOGICAL CONTROL AND MONITORING OF ASEPTIC PROCESSING ENVIRONMENTS

Microbiologically controlled environments are used for a variety of purposes within the healthcare industry. This general information chapter provides information and recommendations for environments where the risk of microbial contamination is controlled through aseptic processing. Products manufactured in such environments include pharmaceutical sterile products, bulk sterile drug substances, sterile intermediates, excipients, and, in certain cases, medical devices. Aseptic processing environments are far more critical in terms of patient risk than controlled environments used for other manufacturing operations—for example, equipment and component preparation, limited bioburden control of nonsterile products, and processing of terminally sterilized products. In this chapter, the type of aseptic processing is differentiated by the presence or absence of human operators. An advanced aseptic process is one in which direct intervention with open product containers or exposed product contact surfaces by operators wearing conventional cleanroom garments is not required and never permitted. [NOTE—A description of terms used in this chapter can be found in the *Glossary* at the end of the chapter.]

The guidance provided in this chapter and the monitoring parameters given for microbiological evaluation should be applied only to clean rooms, restricted-access barrier systems (RABS), and isolators used for aseptic processing. ISO-classified environments used for other purposes are not required to meet the levels of contamination control required for aseptically produced sterile products. The environments used for nonsterile applications require different microbial control strategies.

A large proportion of products labeled as sterile are manufactured by aseptic processing rather than terminal sterilization. Because aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention of microor-

ganisms from entering open containers during processing, product bioburden as well as the bioburden of the manufacturing environment are important factors governing the risk of unacceptable microbial contamination. The terms *aseptic* and *sterile* are not synonymous. *Sterile* means having a complete absence of viable microorganisms or organisms that have the potential to reproduce. In the purest microbiological sense, an *aseptic* process is one that prevents contamination by the exclusion of microorganisms. In contemporary aseptic healthcare-product manufacturing, *aseptic* describes the process for handling sterilized materials in a controlled environment designed to maintain microbial contamination at levels known to present minimal risk.

In any environment where human operators are present, microbial contamination at some level is inevitable. Even the most cautious clean-room environment design and operation will not eliminate the shedding of microorganisms if human operators are present. Thus, an expectation of zero contamination at all locations during every aseptic processing operation is technically not possible and thus is unrealistic. There are no means to demonstrate that an aseptic processing environment and the product-contact surfaces within that environment are sterile. Monitoring locations should be determined based upon a assessment of risk. Although manufacturers should review environmental monitoring results frequently to ensure that the facility operates in a validated state of control, monitoring results can neither prove nor disprove sterility. Because of the limitations of monitoring, manufacturers cannot rely directly on monitoring, statistics, or periodic aseptic-processing simulations to ensure a sterility assurance level.

Environmental monitoring is usually performed by personnel and thus requires operator intervention. As a result, environmental monitoring can both increase the risk of contamination and also give false-positive results. Thus, intensive monitoring is unwarranted, particularly in the ISO 5 environments that are used in the most critical zones of aseptic processing.

A number of sampling methods can be used to assess and control the microbiological status of controlled environments for aseptic processing. At present, nearly all of these methods rely on the growth and recovery of microorganisms, many of which can be in a damaged state caused by environmental stress and therefore may be difficult to recover. The numerical values for air, surface, and personnel monitoring included in this chapter are not intended to represent limits or specifications but are strictly informational. Because of the variety of microbiological sampling equipment and methods, it is not scientifically reasonable to suggest that the attainment of these values guarantees microbial control or that excursions beyond values in this chapter indicate a loss of control. The assessment of risks associated with manufacturing environments must be made over a significant period; and in each case, the contamination recovery rate metric should be established on the basis of a review of actual findings within the facility. The objective of each user should be to use contamination recovery rates to track ongoing performance and to refine the microbiological control program to foster improvements. When optimum operational conditions are achieved within a facility, contamination recovery rate levels typically become relatively stable within a normal range of variability.

There are no standard methods for air sampling, and available literature indicates that air-sampling methods are highly variable. It should not be assumed that similar sample volumes taken by different methods will produce similar rates of recovery. Many factors can affect microbial recovery and survival, and different air sampler suppliers may have designed their systems to meet different requirements. Also, sample-to-sample variation in microbial sampling can be extensive. Limited data are available regarding the accuracy, precision, sensitivity, and limits of detection of monitoring methods used in the aseptic processing of healthcare products.

Surface sampling methods are also not standardized. Different media are employed, and in the case of swabs, different results have been reported for wet and dry swab methods and contact plates. Replicate sample contact plates should be expected to give similar results under identical conditions, but rates of recovery have been reported to be both lower than expected and highly variable. In general, surface monitoring has been found to recover <50%, even when used with relatively high inoculum levels on standardized coupons. In actual production environments where organisms are stressed to varying degrees, recovery rates may be lower.

ADVANCED ASEPTIC TECHNOLOGIES

Advanced aseptic technologies can be defined as those that do not rely on the direct intervention of human operators during processing. At present, technologies such as isolators, blow/fill/seal, and closed RABS (designs that are never opened during setup or operation) may be considered advanced aseptic technologies, provided that direct intervention by gowned personnel is disallowed during processing. In recent years, isolator technology has found a broad acceptance in healthcare manufacturing. Isolators and closed RABS effectively separate the operator from the critical aseptic processing environment. Because these systems substantially reduce contamination risk, their microbiological control levels are higher than those of conventional clean rooms that have comparable particulate air classification level, for example, ISO 5.

CLEAN ROOM CLASSIFICATION FOR ASEPTIC PROCESSING ENVIRONMENTS

The design and construction of clean rooms and controlled environments are covered in ISO 14644 series. This standard defines the performance of a clean environment with respect to the concentration of total particulates per unit volume. ISO 14644-1 stipulates the total particulate counts allowed for a clean environment to meet the defined air quality classifications. The reader is referred to this standard regarding the design characteristics and certification of clean environments.

Pharmaceutical manufacturers are concerned with nonviable particulate contamination in injectable products (see *Particulate Matter in Injections* ⟨788⟩). Unlike microbial contamination in which experimental data suggest that humans are the only significant source, nonviable particulates can arise both from humans and from processing equipment. Studies indicate that gowned humans slough particulate and microbial contamination at a rather consistent rate. However, the relationship between microbial (viable) and nonviable contamination does not hold for particulates shed by processing equipment. Where equipment is the primary source of particulate matter, the resulting particulates are essentially all nonviable.

The argument that if fewer total particulates are present in a clean room, it is less likely that airborne microorganisms will be present is true only if human operators are the source of particulate matter. It is not possible to clearly distinguish between background total particulate contamination generated largely by mechanical operations and the total particulates contributed by personnel. Thus, it is both commonplace and proper for clean-room environmental monitoring programs to consist of both a total particulate component and a microbiological component. *Table 1* describes the clean room classifications commonly used in the pharmaceutical industry. In aseptic processing, clean environments of ISO 14644-1 Classes 5–8 are typically used.

Table 1. Airborne Total Particulate Cleanliness Classes[a]

ISO Class[b]	Particles ≥0.5 μm/m³
ISO 5	3520
ISO 6	35,200
ISO 7	352,000
ISO 8	3,520,000

[a] Taken from ISO International Standard 14644 Part 1, published by the International Organization for Standardization, May 1999.
[b] The four ISO 14644-1 classes correspond closely to former U.S. Federal Standard 209E classifications. The relationships are ISO 5/Class 100, ISO 6/Class 1000, ISO 7/Class 10,000, and ISO 8/Class 100,000.

Isolators and closed RABS present a different picture, because personnel are excluded from the aseptic processing environment and manipulations are made using glove-and-sleeve assemblies and half-suits made of thick, flexible plastic (such as polyvinyl chloride or synthetic rubber). Personnel have far less effect on the microbial quality of the environment within an isolator enclosure than in clean room environments. Some users have chosen to operate RABS in a manner that allows open, direct human intervention. In an open operational state, these systems are more similar in operation to conventional clean rooms and therefore cannot be considered advanced aseptic processing systems. In an open RABS, the ability of operators to adversely affect microbial contamination risk is higher than with closed RABS or isolators.

Specifications for air changes per hour and air velocities are not included in ISO 14644, nor were they included in Federal Standard 209E. Typically, ISO Class 8/Class 100,000 rooms are designed to provide a minimum of 20 air changes per hour; ISO Class 7/Class 10,000 rooms are designed to provide more than 50 air changes per hour; and ISO Class 5/Class 100 clean rooms provide more than 100 air changes per hour. The design of some facility criteria may differ. By diluting and removing contaminants, large volumes of air are likely to reduce airborne contamination in aseptic production. Optimum conditions vary considerably, depending on process characteristics, particularly the amount of contamination derived from personnel. These specifications should be used only as a guide in the design and operation of clean rooms, because the precise correlations among air changes per hour, air velocity, and microbial control have not been satisfactorily established experimentally.

Manufacturers should maintain a predominantly unidirectional flow of air (either vertical or horizontal) in a staffed Class 5 clean room environment, particularly when products, product containers, and closures are exposed. In the evaluation of air movement within a clean room, studying airflow visually by smoke studies or other suitable means is probably more useful than using absolute measures of airflow velocity and change rates. Risk assessment models are another useful way of reducing contamination risk and should be considered.

Air velocity and change rates are far less important in isolators or closed RABS than in clean rooms because personnel are more carefully separated from the product, product containers, and closures. Air velocities substantially lower than those used in human-scale clean rooms have proved adequate in isolator systems and may be appropriate in RABS as well. In zones within isolators where particulate matter poses a hazard to product quality, predominantly vertical or horizontal unidirectional airflow can be maintained. Experience has shown that well-controlled mixing or turbulent airflow is satisfactory for many aseptic processes and for sterility testing within isolators (see *Sterility Testing—Validation of Isolator Systems* ⟨1208⟩).

IMPORTANCE OF A MICROBIOLOGICAL EVALUATION PROGRAM FOR CONTROLLED ENVIRONMENTS

Monitoring of total particulate count in controlled environments, even with the use of electronic instrumentation on a continuous basis, does not provide information on the microbiological content of the environment. The basic limitation of particulate counters is that they measure particles of 0.5 μm or larger. While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of 10–20 μm. Particulate counts as well as microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling. Monitoring the environment for nonviable particulates and microorganisms is an important control function because they both are important in achieving product compendial requirements for *Foreign and Particulate Matter* and *Sterility* in *Injections and Implanted Drug Products* ⟨1⟩.

Total particulate monitoring may provide a better means of evaluating the overall quality of the environment in isolators and closed RABS than in most conventional clean rooms. The superior exclusion of human-borne contamination provided by an isolator results in an increased proportion of nonviable particulates. Total particulate counting in an isolator is likely to provide an immediate indicator of changes in contamination level. Microbial monitoring programs should assess the effectiveness of cleaning and sanitization practices by and of personnel who could have an impact on the bioburden. Because isolators are typically decontaminated using an automatic vapor or gas generation system, microbial monitoring is much less important in establishing their efficiency in eliminating bioburden. These automatic decontamination systems are validated directly, using an appropriate biological indicator challenge, and are controlled to defined exposure parameters during routine use to ensure consistent decontamination.

Microbial monitoring cannot and need not identify and quantify all microbial contaminants in these controlled environments. Microbiological monitoring of a clean room is technically a semiquantitative exercise, because a truly quantitative evaluation of the environment is not possible, given the limitations in sampling equipment. Both the lack of precision of enumeration methods and the restricted sample volumes that can be effectively analyzed suggest that environmental monitoring is incapable of providing direct quantitative information about sterility assurance. Analysts should remember that no microbiological sampling plan can prove the absence of microbial contamination, even when no viable contamination is recovered. The absence of growth on a microbiological sample means only that growth was not discovered; it does not mean that the environment is free of contamination.

Routine microbial monitoring should provide sufficient information to demonstrate that the aseptic processing environment is operating in an adequate state of control. The real value of a microbiological monitoring program lies in its ability to confirm consistent, high-quality environmental conditions at all times. Monitoring programs can detect changes in the contamination recovery rate that may be indicative of changes in the state of control within the environment.

Environmental microbial monitoring and analysis of data by qualified personnel can assist in ensuring that a suitable state of control is maintained. The environment should be sampled during normal operations to allow the collection of meaningful, process-related data. Microbial sampling should occur when materials are in the area, processing activities are ongoing, and a full complement of personnel is working within the aseptic processing environment.

Microbial monitoring of manufacturing clean rooms, RABS, and isolators should include compressed gases, surfaces, room or enclosure air, and any other materials and equipment that might produce a risk of contamination. The analysis of contamination trends in an aseptic environment has long been a component of the environmental control program. In aseptic processing environments and particularly in ISO Class 5 environments, contamination is infrequently observed. In isolator enclosures, contamination is rarer still because of superior exclusion of human-borne contamination. Because of the criticality of these environments, even minor changes in the contamination incident rates may be significant, and manufacturers should frequently and carefully review monitoring data. In less critical environments, microbial contamination may be higher, but changes in recovery rates should be noted, investigated, and corrected. Isolated recoveries of microorganisms should be considered a normal phenomenon in conventional clean rooms, and these incidents generally do not require specific corrective action, because it is almost certain that investigations will fail to yield a scientifically verifiable cause. Because sampling itself requires an aseptic intervention in conventional clean rooms, any single uncorrelated contamination event could be a false positive.

When contamination recovery rates increase from an established norm, process and operational investigation should take place. Investigations will differ depending on the type and processing of the product manufactured in the clean room, RABS, or isolator. Investigation should include a review of area maintenance documentation; sanitization/decontamination documentation; the occurrence of nonroutine events; the inherent physical or operational parameters, such as changes in environmental temperature and relative humidity; and the training status of personnel.

In closed RABS and isolator systems, the loss of glove integrity or the accidental introduction of material that has not been decontaminated are among the most probable causes of detectable microbial contamination. Following the investigation, actions should be taken to correct or eliminate the most probable causes of contamination. Because of the relative rarity of contamination events in modern facilities, the investigation often proves inconclusive. When corrective actions are undertaken, they may include reinforcement of personnel training to emphasize acceptable gowning and aseptic techniques and microbial control of the environment. Some additional microbiological sampling at an increased frequency may be implemented, but this may not be appropriate during aseptic processing because intrusive or overly intensive sampling may entail an increased contamination risk. When additional monitoring is desirable, it may be more appropriate during process simulation studies. Other measures that can be considered to better control microbial contamination include additional sanitization, use of different sanitizing agents, and identification of the microbial contaminant and its possible source.

In any aseptic environment, conventional or advanced, the investigation and the rationale for the course of action chosen as a result of the investigation must be carefully and comprehensively documented.

PHYSICAL EVALUATION OF CONTAMINATION CONTROL EFFECTIVENESS

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation of clean rooms vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a structured approach to physical risk assessment may be appropriate.

One such method has been developed by Ljundqvist and Reinmüller. This method, known as the L-R method, challenges the air ventilation system by evaluating both airflow and the ability of an environment to dilute and remove airborne particles. In the L-R method, a smoke generator allows analysts to visualize the air movements throughout a clean room or a controlled environment, including vortices or turbulent zones, and the airflow pattern can be fine-tuned to minimize these undesirable effects. Following visual optimization of airflow, particulate matter is generated close to the critical zone and sterile field. This evaluation is done under simulated production conditions but with equipment and personnel in place. This type of test can also be used to evaluate the ability of RABS and isolator systems, particularly around product exit ports in these systems, to resist the effects of contamination.

Visual evaluation of air movement within clean rooms is a subjective process. Complete elimination of turbulence or vortices is not possible in operationing clean rooms that contain personnel and equipment. Air visualization is simply one step in the effort to optimize clean room operations and is not a definitive pass/fail test, because acceptable or unacceptable conditions are not readily definable.

Proper testing and optimization of the physical characteristics of the clean room, RABS, or isolator are essential before implementation of the microbiological monitoring program. Assurance that the clean room, RABS, or isolator is in compliance with its predetermined engineering specifications provides confidence that the ability of the facility systems and operating practices to control the bioburden and nonviable particulate matter are appropriate for the intended use. These tests should be repeated during routine certification of the clean room or advanced aseptic processing systems, and whenever significant changes are made to the operation, such as personnel flow, equipment operation, material flow, air-handling systems, or equipment layout.

TRAINING OF PERSONNEL

Good personnel performance plays an essential role in the control of contamination, proper training and supervision are central to contamination control. Aseptic processing is the most critical activity conducted in microbiological controlled environments, and manufacturers must pay close attention to details in all aspects of this endeavor. Rigorous discipline and strict supervision of personnel are essential in order to ensure a level of environmental quality appropriate for aseptic processing.

Training of all personnel working in controlled environments is critical. This training is equally important for personnel responsible for the microbial monitoring program, because contamination of the clean working area could inadvertently occur during microbial sampling. In highly automated operations, monitoring personnel may be the employees who have the most direct contact with the critical surfaces and zones within the processing area. Microbiological sampling has the potential to contribute to microbial contamination caused by inappropriate sampling techniques or by placing personnel in or near the critical zone. A formal training program is required to minimize this risk. This training should be documented for all personnel who enter controlled environments. Interventions should always be minimized, including those required for monitoring activities; but when interventions cannot be avoided, they must be conducted with aseptic technique that approaches perfection as closely as possible.

Management of the facility must ensure that personnel involved in operations in clean rooms and advanced aseptic processing environments are well versed in relevant microbiological principles. The training should include instruction about the basic principles of aseptic technique and should emphasize the relationship of manufacturing and handling procedures to potential sources of product contamination. Those supervising, auditing, or inspecting microbiological control and monitoring activities should be knowledgeable about the basic principles of microbiology, microbial physiology, disinfection and sanitation, media selection and preparation, taxonomy, and sterilization. The staff responsible for supervision and testing should have academic training in medical or environmental microbiology. Sampling personnel as well as individuals working in clean rooms should be knowledgeable about their responsibilities in minimizing the release of microbial contamination. Personnel involved in microbial identification require specialized training about required laboratory methods. Additional training about the management of collected data must be provided. Knowledge and understanding of applicable standard operating procedures are critical, especially those procedures relating to corrective measures taken when environmental conditions require. Understanding of contamination control principles and each individual's responsibilities with respect to good manufacturing practices (GMPs) should be an integral part of the training program, along with training in conducting investigations and in analyzing data.

The only significant sources of microbial contamination in aseptic environments are the personnel. Because operators disperse contamination and because the ultimate objective in aseptic processing is to reduce end-user risk, only healthy individuals should be permitted access to controlled environments. Individuals who are ill must not be allowed to enter an aseptic processing environment, even one that employs advanced aseptic technologies such as isolators, blow/fill/seal, or closed RABS.

The importance of good personal hygiene and a careful attention to detail in aseptic gowning cannot be overemphasized. Gowning requirements differ depending on the use of the controlled environment and the specifics of the gowning system itself. Aseptic processing environments require the use of sterilized gowns with the best available filtration properties. The fullest possible skin coverage is desirable, and sleeve covers or tape should be considered to minimize leaks at the critical glove–sleeve junction. Exposed skin should never be visible in conventional clean rooms under any conditions. The personnel and gowning considerations for RABS are essentially identical to those for conventional clean rooms.

Once employees are properly gowned, they must be careful to maintain the integrity of their gloves, masks, and other gown materials at all times. Operators who work with isolator systems are not required to wear sterilized clean-room gowns, but inadequate aseptic technique and employee-borne contamination are the principal hazards to safe aseptic operations in isola-

tors, as well as RABS, and in conventional clean rooms. Glove-and-sleeve assemblies can develop leaks that can allow the mechanical transfer of microorganisms to the product. A second glove, worn either under or over the primary isolator/RABS glove, can provide an additional level of safety against glove leaks or can act as a hygienic measure. Also, operators must understand that aseptic technique is an absolute requirement for all manipulations performed with gloves within RABS and isolator systems.

The environmental monitoring program, by itself, cannot detect all events in aseptic processing that might compromise the microbiological quality of the environment. Therefore, periodic media-fill or process simulation studies are necessary, as is thorough ongoing supervision, to ensure that appropriate operating controls and training are effectively maintained.

CRITICAL FACTORS IN THE DESIGN AND IMPLEMENTATION OF A MICROBIOLOGICAL ENVIRONMENTAL MONITORING PROGRAM

Since the advent of comprehensive environmental monitoring programs, their applications in capturing adverse trends or drifts has been emphasized. In a modern aseptic processing environment—whether an isolator, RABS, or conventional clean room—contamination has become increasingly rare. Nevertheless, a monitoring program should be able to detect a change from the validated state of control in a facility and to provide information for implementing appropriate countermeasures. An environmental monitoring program should be tailored to specific facilities and conditions. It is also helpful to take a broad perspective in the interpretation of data. A single uncorrelated result on a given day may not be significant in the context of the technical limitations associated with aseptic sampling methods.

Selection of Growth Media

A general microbiological growth medium such as soybean–casein digest medium (SCDM) is suitable for environmental monitoring in most cases because it supports the growth of a wide range of bacteria, yeast, and molds. This medium can be supplemented with additives to overcome or to minimize the effects of sanitizing agents or of antibiotics. Manufacturers should consider the specific detection of yeasts and molds. If necessary, general mycological media such as Sabouraud's, modified Sabouraud's, or inhibitory mold agar can be used. In general, monitoring for strict anaerobes is not performed, because these organisms are unlikely to survive in ambient air. However, micro-aerophilic organisms may be observed in aseptic processing. Should anoxic conditions exist or if investigations warrant (e.g., identification of these organisms in sterility testing facilities or *Sterility Tests* ⟨71⟩ results), monitoring for micro-aerophiles and organisms that grow under low-oxygen conditions may be warranted. The ability of any media used in environmental monitoring, including those selected to recover specific types of organisms, must be evaluated for their ability to support growth, as indicated in ⟨71⟩.

Selection of Culture Conditions

Time and incubation temperatures are set once the appropriate media have been selected. Typically, for general microbiological growth media such as SCDM, incubation temperatures in the ranges of approximately 20°–35° have been used with an incubation time of not less than 72 hours. Longer incubation times may be considered when contaminants are known to be slow growing. The temperature ranges given above are by no means absolute. Mesophilic bacteria and mold common to the typical facility environment are generally capable of growing over a wide range of temperatures. For many mesophilic organisms, recovery is possible over a range of approximately 20°. In the absence of confirmatory evidence, microbiologists may incubate a single plate at both a low and a higher temperature. Incubating at the lower temperature first may compromise the recovery of Gram-positive cocci that are important because they are often associated with humans.

Sterilization processes for preparing growth media should be validated. When selective media are used for monitoring, incubation conditions should reflect published technical requirements. Contamination should not be introduced into a manufacturing clean room as a result of using contaminated sampling media or equipment. Of particular concern is the use of aseptically prepared sampling media. Wherever possible, sampling media and their wrappings should be terminally sterilized by moist heat, radiation, or other suitable means. If aseptically prepared media must be used, analysts must carry out preincubation and visual inspection of all sampling media before introduction into the clean room. The reader is referred to *Microbiological Best Laboratory Practices* ⟨1117⟩ for further information regarding microbiology laboratory operations and control.

ESTABLISHMENT OF SAMPLING PLAN AND SITES

During initial startup or commissioning of a clean room or other controlled environment, specific locations for air and surface sampling should be determined. Locations considered should include those in proximity of the exposed product, containers, closures, and product contact surfaces. In aseptic processing, the area in which containers, closures, and product are exposed to the environment is often called the *critical zone*—the critical zone is always ISO 5. For aseptic operations the entire critical zone should be treated as a sterile field. A nonsterile object, including the gloved hands of clean room personnel or an RABS/isolator glove, should never be brought into contact with a sterile product, container closure, filling station, or conveying equipment before or during aseptic processing operations. Operators and environmental monitoring personnel should never

touch sterile parts of conveyors, filling needles, parts hoppers, or any other equipment that is in the product-delivery pathway. This means that surface monitoring on these surfaces is best done at the end of production operations.

The frequency of sampling depends on the manufacturing process conducted within an environment. Classified environments that are used only to provide a lower overall level of bioburden in nonsterile product manufacturing areas require relatively infrequent environmental monitoring. Classified environments in which closed manufacturing operations are conducted, including fermentation, sterile API processing, and chemical processes, require fewer monitoring sites and less frequent monitoring because the risk of microbial contamination from the surrounding environment is comparatively low. Microbiological monitoring of environments in which products are filled before terminal sterilization is generally less critical than the monitoring of aseptic processing areas. The amount of monitoring required in filling operations for terminal sterilization depends on the susceptibility of the product survival and the potential for proliferation of microbial contamination. The identification and estimated number of microorganisms that are resistant to the subsequent sterilization may be more critical than the microbiological monitoring of the surrounding manufacturing environments.

It is not possible to recommend microbial control levels for each type of manufacturing environment. The levels established for one ISO Class 7 environment, for example, may be inappropriate for another ISO Class 7 environment, depending on the production activities undertaken in each. The user should conduct a prospective risk analysis and develop a rationale for the sampling locations and frequencies for each controlled environment. The classification of a clean room helps establish control levels, but that does not imply that all rooms of the same classification should have the same control levels and the same frequency of monitoring. Monitoring should reflect the microbiological control requirements of manufacturing or processing activities. Formal risk assessment techniques can result in a scientifically valid contamination control program.

Table 2 suggests frequencies of sampling in decreasing order of frequency and in relation to the criticality or product risk of the area being sampled. This table distinguishes between aseptic processing where personnel are aseptically gowned and those where a lesser gowning is appropriate. Environmental monitoring sampling plans should be flexible with respect to monitoring frequencies, and sample plan locations should be adjusted on the basis of the observed rate of contamination and ongoing risk analysis. On the basis of long-term observations, manufacturers may increase or decrease sampling at a given location or eliminate a sampling location altogether. Oversampling can be as deleterious to contamination control as undersampling, and careful consideration of risk and reduction of contamination sources can guide the sampling intensity.

Table 2. Suggested Frequency of Sampling for Aseptic Processing Areas[a]

Sampling Area/Location	Frequency of Sampling
Clean Room/RABS	
Critical zone (ISO 5 or better)	
Active air sampling	Each operational shift
Surface monitoring	At the end of the operation
Aseptic area adjacent critical zone	
All sampling	Each operating shift
Other nonadjacent aseptic areas	
All sampling	Once per day
Isolators	
Critical zone (ISO 5 or better)	
Active air sampling	Once per day
Surface monitoring	At the end of the campaign
Nonaseptic areas surrounding the isolator	
All sampling	Once per month

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

SELECTION OF SAMPLE SITES WITHIN CLEAN ROOMS AND ASEPTIC PROCESSING AREAS

ISO 14644 suggests a grid approach for the total particulate air classification of clean rooms. This approach is appropriate for certifying the total particulate air quality performance against its design objective. Grids may also have value in analyzing risk from microbial contamination, although in general, grids that have no personnel activity are likely to have low risk of contamination. Microbial contamination is strongly associated with personnel, so microbiological monitoring of unstaffed environments is of limited value.

Microbiological sampling sites are best selected with consideration of human activity during manufacturing operations. Careful observation and mapping of the clean room during the qualification phase can provide useful information concerning the movement and positioning of personnel. Such observation can also yield important information about the most frequently conducted manipulations and interventions.

The location and movement of personnel within the clean room correlate with contamination risk to the environment and to the processes conducted within that environment. Sample sites should be selected so that they evaluate the impact of personnel movement and work within the area, particularly interventions and manipulations within the critical zone.

The most likely route of contamination is airborne, so the samples most critical to risk assessment are those that relate to airborne contamination near exposed sterile materials. Other areas of concern are entry points where equipment and materials move from areas of lower classification to those of higher classification. Areas within and around doors and airlocks should be included in the monitoring scheme. It is customary to sample walls and floors, and indeed sampling at these locations can provide information about the effectiveness of the sanitization program. Sampling at these locations can take place relatively infrequently, because contamination there is unlikely to affect product. Operators should never touch floors and walls, so mechanical transmission of contamination from these surfaces to critical areas where product is exposed should not occur.

Manufacturers typically monitor surfaces within the critical zone, although this should be done only at the end of operations. Residues of media or diluent from wet swabs should be avoided on surfaces, because they could lead to microbial proliferation. Also, cleaning surfaces to remove diluent or media requires personnel intervention and movements that can result in release of microbial contamination into the critical zone and can disrupt airflow.

MICROBIOLOGICAL CONTROL PARAMETERS IN CLEAN ROOMS, ISOLATORS, AND RABS

Since the early 1980s, manufacturers have established alert and action levels for environmental monitoring. In recent years the numerical difference between alert and action levels has become quite small, especially in ISO 5 environments. Growth and recovery in microbiological assays have normal variability in the range of $\pm 0.5 \log_{10}$. Studies on active microbiological air samplers indicate that variability of as high as tenfold is possible among commonly used sampling devices. As a result of this inherent variability and indeterminate sampling error, the supposed differences between, for example, an alert level of 1 cfu and an action level of 3 cfu are not analytically significant. Treating differences that are within expected, and therefore, normal ranges as numerically different is not scientifically valid and can result in unwarranted activities. In a practical sense, numerical values that vary by as much as five- to tenfold may not be significantly different.

Because of the limited accuracy and precision of microbial growth and recovery assays, analysts can consider the frequency with which contamination is detected rather than absolute numbers of cfu detected in any single sample. Also, a cfu is not a direct enumeration of microorganisms present but rather is a measure of contamination that may have originated from a clump of organisms.

Mean contamination recovery rates should be determined for each clean room environment, and changes in contamination recovery rate at a given site or within a given room may indicate the need for corrective action. Within the ISO 5 critical zone, airborne and surface contamination recovery rates of 1% or less should be attainable with current methods. Contamination recovery rates for closed RABS and isolator systems should be significantly lower still and can be expected to be <0.1%, on the basis of published monitoring results.

Contamination observed at multiple sites in an environment within a single sampling period may indicate increased risk to product and should be carefully evaluated. The appearance of contamination nearly simultaneously at multiple sites could also arise from poor sampling technique, so careful review is in order before drawing conclusions about potential loss of control. Resampling an environment several days after contamination is of little value, because the conditions during one sampling occasion may not be accurately duplicated during another.

Surface samples may also be taken from clean room garments. Personnel sampling should be emphasized during validation and is best done at the completion of production work in order to avoid adventitious contamination of the garments. In this case the average should be <1% for these sample sites as well. Gloves on closed RABS and isolators should meet the more rigorous expectation of <0.1% contamination recovery rates.

Because of the inherent variability of microbial sampling methods, contamination recovery rates are a more useful measure of trending results than is focusing on the number of colonies recovered from a given sample. *Table 3* provides recommended contamination recovery rates for aseptic processing environments. The incident rate is the rate at which environmental samples are found to contain microbial contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number. In other words, 99% of the samples taken are completely free of contamination. Contamination recovery rates that are higher than those recommended in *Table 3* may be acceptable in rooms of similar classification that are used for lower-risk activities. Action should be required when the contamination recovery rate trends above these recommendations for a significant time.

Table 3. Suggested Initial Contamination Recovery Rates in Aseptic Environments[a]

Room Classification	Active Air Sample (%)	Settle Plate (9 cm) 4 h Exposure (%)	Contact Plate or Swab (%)	Glove or Garment (%)
Isolator/Closed RABS (ISO 5 or better)	<0.1	<0.1	<0.1	<0.1
ISO 5	<1	<1	<1	<1
ISO 6	<3	<3	<3	<3

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

General Chapters

Table 3. Suggested Initial Contamination Recovery Rates in Aseptic Environments[a] *(Continued)*

Room Classification	Active Air Sample (%)	Settle Plate (9 cm) 4 h Exposure (%)	Contact Plate or Swab (%)	Glove or Garment (%)
ISO 7	<5	<5	<5	<5
ISO 8	<10	<10	<10	<10

[a] All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

Detection frequency should be based on actual monitoring data and should be retabulated monthly. Action levels should be based on empirical process capability. If detection frequencies exceed the recommendations in *Table 3* or are greater than established process capability, then corrective actions should be taken. Corrective actions may include but are not limited to the following:

- Revision of the sanitization program, including selection of antimicrobial agents, application methods, and frequencies
- Increased surveillance of personnel practices, possibly including written critiques of aseptic methods and techniques
- Review of microbiological sampling methods and techniques

When higher-than-typical recovery levels for glove and garment contamination are observed, additional training for gowning practices may be indicated.

SIGNIFICANT EXCURSIONS

Excursions beyond approximately 15 cfu recovered from a single ISO 5 sample, whether from airborne, surface, or personnel sources, should happen very infrequently. When such ISO 5 excursions do occur, they may be indicative of a significant loss of control when they occur within the ISO 5 critical zone in close proximity to product and components. Thus, any ISO 5 excursion >15 cfu should prompt a careful and thorough investigation.

A key consideration for an abnormally high number of recovered colonies is whether this incident is isolated or can be correlated with other recoveries. Microbiologists should review recovery rates for at least two weeks before the incident of abnormally high recovery so that they can be aware of other recoveries that might indicate an unusual pattern. Microbiologists should carefully consider all recoveries, including those that are in the more typical range of 1–5 cfu. The identity of the organisms recovered is an important factor in the conduct of this investigation.

In the case of an isolated single excursion, establishing a definitive cause probably will not be possible, and only general corrective measures can be considered. It is never wise to suggest a root cause for which there is no solid scientific evidence. Also, there should be an awareness of the variability of microbial analysis. Realistically, there is no scientific reason to treat a recovery of 25 cfu as statistically different from a recovery of 15 cfu. A value of 15 cfu should not be considered significant in terms of process control, because realistically there is no difference between a recovery of 14 cfu and one of 15 cfu. Microbiologists should use practical scientific judgment in their approach to excursions.

FURTHER CONSIDERATIONS ABOUT DATA INTERPRETATION

In the high-quality environments required for aseptic processing, detection frequency typically is low. As can be seen from the rates recommended in *Table 3*, the majority of samples taken in an aseptic processing area will yield a recovery of zero contamination. In the most critical areas within an aseptic processing operation, it is expected that less than 1% of the samples will yield any recoverable contamination. In the most advanced of modern aseptic operations that use separative technologies such as isolators or closed RABS, the recovery rate will approach zero at all times.

The microbiologist responsible for environmental control or sterility assurance should not take this to mean that the environmental quality approaches sterility. The sensitivity of any microbial sampling system in absolute terms is not known. In environmental monitoring, a result of zero means only that the result is below the limit of detection of the analytical system. A false sense of security should not be derived from the infrequency of contamination recovery in aseptic processing.

Sterility assurance is best accomplished by a focus on human-borne contamination and the facility design features that best mitigate risk from this contamination. Greatest risk mitigation can be attained by reducing or eliminating human interventions through proper equipment design and by providing sufficient air exchanges per hour for the intended personnel population of the facility. Other risk mitigation factors include effective personnel and material movement and the proper control of temperature and humidity. Secondary factors for risk mitigation include cleaning and sanitization. Risk analysis models that analyze processes prospectively to reduce human-borne contamination risk by minimizing operator interventions are more powerful tools for sterility assurance than monitoring. Environmental monitoring cannot prove or disprove in absolute terms the sterility of a lot of product. Environmental monitoring can only assure those responsible for a process that a production system is in a consistent, validated state of control. Care should be taken to avoid drawing inappropriate conclusions from monitoring results.

SAMPLING AIRBORNE MICROORGANISMS

Among the most commonly used tools for monitoring aseptic environments are impaction and centrifugal samplers. A number of commercially available samplers are listed for informational purposes. The selection, appropriateness, and adequacy of using any particular sampler are the responsibility of the user.

Slit-to-Agar Air Sampler (STA)

The unit is powered by an attached source of controllable vacuum. The air intake is obtained through a standardized slit below which is placed a slowly revolving Petri dish that contains a nutrient agar. Airborne particles that have sufficient mass impact the agar surface, and viable organisms are allowed to grow. A remote air intake is often used to minimize disturbance of unidirectional airflow.

Sieve Impactor

This apparatus consists of a container designed to accommodate a Petri dish that contains a nutrient agar. The cover of the unit is perforated with openings of a predetermined size. A vacuum pump draws a known volume of air through the cover, and airborne particles that contain microorganisms impact the agar medium in the Petri dish. Some samplers feature a cascaded series of sieves that contain perforations of decreasing size. These units allow determination of the size range distribution of particulates that contain viable microorganisms based on the size of the perforations through which the particles landed on the agar plates.

Centrifugal Sampler

The unit consists of a propeller or turbine that pulls a known volume of air into the unit and then propels the air outward to impact on a tangentially placed nutrient agar strip set on a flexible plastic base.

Sterilizable Microbiological Atrium

The unit is a variant of the single-stage sieve impactor. The unit's cover contains uniformly spaced orifices approximately 0.25 inch in size. The base of the unit accommodates one Petri dish containing a nutrient agar. A vacuum pump controls the movement of air through the unit, and a multiple-unit control center as well as a remote sampling probe are available.

Surface Air System Sampler

This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit's perforated cover over the agar contact plate and beyond the motor, where it is exhausted. Multiple mounted assemblies are also available.

Gelatin Filter Sampler

The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.

Settling Plates

This method is still widely used as a simple and inexpensive way to qualitatively assess the environments over prolonged exposure times. Published data indicate that settling plates, when exposed for 4- to 5-hour periods, can provide a limit of detection for a suitable evaluation of the aseptic environment. Settling plates may be particularly useful in critical areas where active sampling could be intrusive and a hazard to the aseptic operation.

One of the major drawbacks of mechanical air samplers is the limited sample size of air being tested. When the microbial level in the air of a controlled environment is expected to contain extremely low levels of contamination per unit volume, at least 1 cubic meter of air should be tested in order to maximize sensitivity. Typically, slit-to-agar devices have an 80-L/min sampling capacity (the capacity of the surface air system is somewhat higher). If 1 cubic meter of air were tested, then it would require an exposure time of 15 min. It may be necessary to use sampling times in excess of 15 min to obtain a representative environmental sample. Although some samplers are reported to have high sampling volumes, consideration should be given to the potential for disruption of the airflow patterns in any critical area and to the creation of turbulence.

Technicians may wish to use remote sampling systems in order to minimize potential risks resulting from intervention by environmental samplers in critical zones. Regardless of the type of sampler used, analysts must determine that the extra tubing needed for a remote probe does not reduce the method's sensitivity to such an extent that detection of low levels of contamination becomes unlikely or even impossible.

SURFACE SAMPLING

Another component of the microbial-control program in controlled environments is surface sampling of equipment, facilities, and personnel. The standardization of surface sampling methods and procedures has not been as widely addressed in the pharmaceutical industry as has the standardization of air-sampling procedures. Surface sampling can be accomplished by the use of contact plates or by the swabbing method.

Contact plates filled with nutrient agar are used for sampling regular or flat surfaces and are directly incubated for the appropriate time and temperature for recovery of viable organisms. Specialized agar can be used for the recovery of organisms that have specific growth requirements. Microbial estimates are reported per contact plate.

The swabbing method can be used to supplement contact plates for sampling of irregular surfaces, especially irregular surfaces of equipment. The area that will be swabbed is defined with a sterile template of appropriate size. In general, it is in the range of 24–30 cm^2. After sample collection the swab is placed in an appropriate diluent or transport medium and is plated onto the desired nutrient agar. The microbial estimates are reported per swab of defined sampling area.

Surface monitoring is used as an environmental assessment tool in all types of classified environments. In ISO 5 environments for aseptic processing, surface monitoring is generally performed beside critical areas and surfaces. Component hoppers and feed chutes that contact sterile surfaces on closures and filling needles can be tested for microbial contamination. Often in conventional staffed clean rooms, these product contact surfaces are steam sterilized and aseptically assembled. The ability of operators to perform these aseptic manipulations are evaluated during process stimulations or media fills, although true validation of operator technique in this manner is not possible. Surface monitoring on surfaces that directly contact sterile parts or product should be done only after production operations are completed. Surface sampling is not a sterility test and should not be a criterion for the release or rejection of product. Because these samples must be taken aseptically by personnel, it is difficult to establish with certainty that any contamination recovered is product related.

CULTURE MEDIA AND DILUENTS

The type of medium, liquid or solid, used for sampling or plating microorganisms depends on the procedure and equipment used. Any medium used should be evaluated for suitability for the intended purpose. The most commonly used all-purpose solid microbiological growth medium is soybean–casein digest agar. As previously noted, this medium can be supplemented with chemicals that counteract the effect of various antimicrobials.

IDENTIFICATION OF MICROBIAL ISOLATES

A successful environmental control program includes an appropriate level of identification of the flora obtained by sampling. A knowledge of the flora in controlled environments aids in determining the usual microbial flora anticipated for the facility and in evaluating the effectiveness of the cleaning and sanitization procedures, methods, agents, and recovery methods. The information gathered by an identification program can be useful in the investigation of the source of contamination, especially when recommended detection frequencies are exceeded.

Identification of isolates from critical and immediately adjacent areas should take precedence over identification of microorganisms from noncritical areas. Identification methods should be verified, and ready-to-use kits should be qualified for their intended purpose.

CONCLUSION

Environmental monitoring is one of several key elements required in order to ensure that an aseptic processing area is maintained in an adequate level of control. Monitoring is a qualitative exercise, and even in the most critical applications such as aseptic processing, conclusions regarding lot acceptability should not be made on the basis of environmental sampling results alone. Environments that are essentially free of human operators generally have low initial contamination rates and maintain low levels of microbial contamination. Human-scale clean rooms present a very different picture. Studies conclusively show that operators, even when carefully and correctly gowned, continuously slough microorganisms into the environment. Therefore, it is unreasonable to assume that samples producing no colonies, even in the critical zone or on critical surfaces, will always be observed. Periodic excursions are a fact of life in human-scale clean rooms, but the contamination recovery rate, particularly in ISO 5 environments used for aseptic processing, should be consistently low.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous improvement of personnel operations and environmental control. In general, fewer per-

sonnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from microbial contamination.

GLOSSARY

Airborne Particulate Count (also referred to as *Total Particulate Count*): The total number of particles of a given size per unit volume of air.

Airborne Viable Particulate Count (also referred to as *Total Airborne Aerobic Microbial Count*): The recovered number of colony-forming units (cfu) per unit volume of air.

Air Changes: The frequency per unit of time (minutes, hours, etc.) that the air within a controlled environment is replaced. The air can be recirculated partially or totally replaced.

Air Sampler: Devices or equipment used to sample a measured amount of air in a specified time to quantitate the particulate or microbiological status of air in the controlled environment.

Aseptic: Technically, the absence of microorganisms, but in aseptic processing this refers to methods and operations that minimize microbial contamination in environments where sterilized product and components are filled and/or assembled.

Aseptic Processing: An operation in which the product is assembled or filled into its primary package in an ISO 5 or better environment and under conditions that minimize the risk of microbial contamination. The ultimate goal is to produce products that are as free as possible of microbial contamination.

Barrier System: Physical barriers installed within an aseptic processing room to provide partial separation between aseptically gowned personnel and critical areas subject to considerable contamination risk. Personnel access to the critical zone is largely unrestricted. It is subject to a high level disinfection.

Bioburden: Total number and identity of the predominant microorganisms detected in or on an article.

Clean Room: A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness Class. In addition, the concentration of microorganisms in the environment is monitored; each cleanliness Class defined is also assigned a microbial level for air, surface, and personnel gear.

Commissioning of a Controlled Environment: Certification by engineering and quality control that the environment has been built according to the specifications of the desired cleanliness Class and that, under conditions likely to be encountered under normal operating conditions (or worst-case conditions), it is capable of delivering an aseptic process. Commissioning includes media-fill runs and results of the environmental monitoring program.

Contamination Recovery Rate: The contamination recovery rate is the rate at which environmental samples are found to contain any level of contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number.

Controlled Environment: Any area in an aseptic process system for which airborne particulate and microorganism levels are controlled to specific levels, appropriate to the activities conducted within that environment.

Corrective Action: Actions to be performed that are according to standard operating procedures and that are triggered when certain conditions are exceeded.

Critical Zone: Typically the entire area where product and the containers and closures are exposed in aseptic processing.

Detection Frequency: The frequency with which contamination is observed in an environment. Typically expressed as a percentage of samples in which contamination is observed per unit of time.

Environmental Isolates: Microorganisms that have been isolated from the environmental monitoring program.

Environmental Monitoring Program: Documented program implemented via standard operating procedures that describes in detail the methods and acceptance criteria for monitoring particulates and microorganisms in controlled environments (air, surface, personnel gear). The program includes sampling sites, frequency of sampling, and investigative and corrective actions.

Equipment Layout: Graphical representation of an aseptic processing system that denotes the relationship between and among equipment and personnel. This layout is used in the *Risk Assessment Analysis* to determine sampling site and frequency of sampling based on potential for microbiological contamination of the product/container/closure system. Changes must be assessed by responsible managers, since unauthorized changes in the layout for equipment or personnel stations could result in increase in the potential for contamination of the product/container/closure system.

Isolator for Aseptic Processing: An aseptic isolator is an enclosure that is over-pressurized with HEPA filtered air and is decontaminated using an automated system. When operated as a closed system, it uses only decontaminated interfaces or rapid transfer ports (RTPs) for materials transfer. After decontamination they can be operated in an open manner with the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. It can be used for aseptic processing activities or for asepsis and containment simultaneously.

Material Flow: The flow of material and personnel entering controlled environments should follow a specified and documented pathway that has been chosen to reduce or minimize the potential for microbial contamination of the product/closure/container systems. Deviation from the prescribed flow could result in increase in the potential for microbial contamination. Material/personnel flow can be changed, but the consequences of the changes from a microbiological point of view should be assessed by responsible managers and must be authorized and documented.

Media Fill: Microbiological simulation of an aseptic process by the use of growth media processed in a manner similar to the processing of the product and with the same container/closure system being used.

Media Growth Promotion: Procedure that references *Growth Promotion Test of Aerobes, Anerobes, and Fungi* in *Sterility Tests* ⟨71⟩ to demonstrate that media used in the microbiological environmental monitoring program, or in *media-fill* runs, are capable of supporting growth of indicator microorganisms and of environmental isolates from samples obtained through the monitoring program or their corresponding ATCC strains.
Product Contact Areas: Areas and surfaces in a controlled environment that are in direct contact with either products, containers, or closures and the microbiological status of which can result in potential microbial contamination of the product/container/closure system.
Restricted Access Barrier System (RABS): An enclosure that relies on HEPA filtered air over-spill to maintain separation between aseptically gowned personnel and the operating environment. It is subject to a high level of disinfection prior to use in aseptic process. It uses decontaminated (where necessary) interfaces or RTPs for materials transfer. It allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. If opened subsequent to decontamination, its performance capability is adversely impacted.
Risk Assessment Analysis: Analysis of the identification of contamination potentials in controlled environments that establish priorities in terms of severity and frequency and that will develop methods and procedures that will eliminate, reduce, minimize, or mitigate their potential for microbial contamination of the product/container/closure system.
Sampling Plan: A documented plan that describes the procedures and methods for sampling a controlled environment; identifies the sampling sites, the sampling frequency, and number of samples; and describes the method of analysis and how to interpret the results.
Sampling Sites: Documented geographical location, within a controlled environment, where sampling for microbiological evaluation is taken. In general, sampling sites are selected because of their potential for product/container–closure contacts.
Standard Operating Procedures: Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place in a controlled environment and auxiliary environments. Deviations from standard operating procedures should be noted and approved by responsible managers.
Sterile or Aseptic Field: In aseptic processing or in other controlled environments, it is the space at the level of or above open product containers, closures, or product itself, where the potential for microbial contamination is highest.
Sterility: Within the strictest definition of sterility, an article is deemed sterile when there is complete absence of viable microorganisms. *Viable*, for organisms, is defined as having the capacity to reproduce. Absolute sterility cannot be practically demonstrated because it is technically unfeasible to prove a negative absolute. Also, absolute sterility cannot be practically demonstrated without testing every article in a batch. Sterility is defined in probabilistic terms, where the likelihood of a contaminated article is acceptably remote.
Swabs for Microbiological Sampling: Devices used to remove microorganisms from irregular or regular surfaces for cultivation to identify the microbial population of the surface. A swab is generally composed of a stick with an absorbent tip that is moistened before sampling and is rubbed across a specified area of the sample surface. The swab is then rinsed in a sterile solution to suspend the microorganisms, and the solution is transferred to growth medium for cultivation of the microbial population.
Trend Analysis: Data from a routine microbial environmental monitoring program that can be related to time, shift, facility, etc. This information is periodically evaluated to establish the status or pattern of that program to ascertain whether it is under adequate control. A trend analysis is used to facilitate decision-making for requalification of a controlled environment or for maintenance and sanitization schedules.

APPENDIX

Additional Resources

Agalloco J, Akers J. Risk analysis for aseptic processing: The Akers-Agalloco method. *Pharm Technol.* 2005; 29(11):74–88.
Agalloco J, Akers J. The simplified Akers-Agalloco method for aseptic processing risk analysis. *Pharm Technol.* 2006; 31(7)60–72.
Akers, J. The proper role of environmental monitoring in aseptic processing. *Am Pharm Rev.* 2006; 9(4):24–28.
CDC, Healthcare Infection Control Advisory Committee. Guidelines for environmental control in healthcare facilities. *MMWR* 2003; 52(No. RR-10):1–42.
Favero MS, Puleo JR, Marshall JH, Oxborrow GS. Microbiological sampling of surfaces. *J Appl Bacteriol.* 1968; 31:336–346.
Hussong D, Madsen R. Analysis of environmental microbiology data from clean room samples. *Pharm Technol.* 2004; Aseptic Processing Suppl:10–15.
International Organization for Standardization (ISO). 14644-1, Clean rooms and associated environments, part 1: classification of air cleanliness. Geneva: ISO; 1999.
International Organization for Standardization (ISO). 14644-2, Clean rooms and associated environments, part 2: specifications for testing and monitoring to prove continued compliance with 14644 part 1. Geneva: ISO; 2000.
Jensen PA, Todd WF, Davis GN, Scarpino PV. Evaluation of eight bioaerosol samplers challenged with aerosol of free bacteria. *Am Ind Hyg Assoc J.* 1992; 53:660–667.
Ljungqvist B. Active sampling of airborne viable particulate in controlled environments: a comparative study of common instruments. *Eur J Parenter Sci.* 1998; 3:59–62.

General Chapters

Ljungqvist B, Reinmüller B. Interaction between air movements and the dispersion of contaminants: clean zones with unidirectional air flow. *J Parenter Sci Technol.* 1993; 47(2):60–69.
Ljungqvist B, Reinmüller B. Airborne viable particles and total number of airborne particles: comparative studies of active air sampling. *PDA J Sci Technol.* 2000; 54:112–116.
Maruyama M, Matsuoka T, Deguchi M, Akers J. The application of robotics to aseptic surface monitoring. *Pharm Technol.* 2007; 32(7):40–44.
Process simulation testing for sterile bulk pharmaceutical chemicals. PDA Technical Report No. 28. *J Parenter Sci Technol.* 1998; 52 S3.
Reinmüller B. Dispersion and risk assessment of airborne contaminants in pharmaceutical cleanrooms. *Building Serv Eng Bull* (Sweden). 2001; Bulletin No. 56.
Stewart SL, Grinshpun SA, Willeke K, Terzieva S, Ulevicius V, Donnelly J. Effect of impact stress on microbial recovery on an agar surface. *Appl Environ Micro.* 1995; 61:1232–1239.
Whyte W. Reduction of microbial dispersion by clothing. *J Parenter Sci Technol.* 1985; 39(1):51–60.

⟨1117⟩ MICROBIOLOGICAL BEST LABORATORY PRACTICES

INTRODUCTION

Good laboratory practices in a microbiology laboratory consist of activities that depend on several principles: aseptic technique, control of media, control of test strains, operation and control of equipment, diligent recording and evaluation of data, and training of the laboratory staff. Because of the inherent risk of variability in microbiology data, reliability and reproducibility are dependent on the use of accepted methods and adherence to good laboratory practices.

MEDIA PREPARATION AND QUALITY CONTROL

Media Preparation

Culture media are the basis for most microbiological tests. Safeguarding the quality of the media is therefore critical to the success of the microbiology laboratory. Media preparation, proper storage, and quality control testing can ensure a consistent supply of high-quality media.

It is important to choose the correct media or components in making media based on the use of accepted sources or references for formulas. The manufacturer's formula and instructions for preparation routinely accompany dehydrated media and ready-made media. Because different media types may have different preparation requirements (e.g., heating, additives, and pH adjustment), it is important to follow these instructions to ensure preparation of acceptable media quality. A certificate of analysis describing expiration dating and recommended storage conditions accompanies ready-made media, as well as the quality control organisms used in growth-promotion and selectivity testing of that media.

Water is the universal diluent for microbiological media. Purified Water is most often used for media preparation, but in certain cases the use of deionized or distilled water may be appropriate. Water of lesser quality should not be used for microbiological media preparation. The volume of the water used should be recorded.

Consistent preparation of media requires accurate weighing of dehydrated media or media constituents. A calibrated balance with the appropriate weight range for the ingredients should be used (See *Weighing on an Analytical Balance* ⟨1251⟩). Clean weighing containers and tools (such as spatulas) should be used to prevent foreign substances from entering the formulation. The weight of the components should be recorded.

Dehydrated media should be thoroughly dissolved in water before dispensing and sterilization. If heating is necessary to help dissolve the media, care should be taken not to overheat media, because all culture media, to a greater or lesser extent, are heat-sensitive. Equipment used in the preparation of media should be appropriate to allow for controlled heating, constant agitation, and mixing of the media. Darkening of media (Maillard-type reaction or nonenzymatic browning) is a general indication of overheating. When adding required supplements to media, adequate mixing of the medium after adding the supplement should be performed.

Preparation of media in poorly cleaned glassware can allow inhibitory substances to enter the media. Inhibitory substances can come from detergent residue after cleaning glassware or from prior materials used in the glassware. Be sure that the cleaning process removes debris and foreign matter, and that the detergent is thoroughly rinsed out with Purified Water. See *Cleaning Glass Apparatus* ⟨1051⟩ for additional guidance.

Sterilization of media should be performed within the parameters provided by the manufacturer or validated by the user. Commercially prepared media should provide documentation of the sterilization method used. Autoclaving by moist heat is the preferred sterilization technique, except in instances when boiling is required in order to avoid deterioration of heat-labile components of the media. Sterilization by filtration may also be appropriate for some formulations.

General Chapters

The effects of the sterilization method and conditions on the media should be validated by sterility and growth-promotion testing of the media. In addition, if sterilized by moist heat, the autoclave cycle should be validated to ensure proper heat distribution for selected loads and volumes. Typically, manufacturers recommend using an autoclave cycle of 121° for 15 minutes using a validated autoclave. These conditions apply to time at temperature of the media. As container size and the load configuration of the autoclave will influence the rate of heating, longer cycles may be required for larger loads. However, the sterilization time will be dependent on the media volume and autoclave load. Sterilization cycles in which the autoclave is slow to come up to temperature may result in overheating of the media. Therefore, care must be taken to validate a sterilization cycle, balancing the need for sterile media against the tendency of the media to degrade under excessive heating. Storage of the media in the autoclave after the liquid cycle is completed is not recommended after cooling, as it may damage the media. Improper heating or sterilizing conditions—for commercially prepared or internally prepared media—may result in a difference in color change, loss of clarity, altered gel strength, or pH drift from the manufacturer's recommended range, as well as reduced growth-promotion activity and/or selectivity.

The pH of each batch of medium should be confirmed after it has cooled to room temperature (20°–25°) by aseptically withdrawing a sample for testing. Refrigerated purchased media should be allowed to warm up to ambient room temperature if it is to be checked for pH confirmation. A flat pH probe is recommended for agar surfaces, and an immersion probe is recommended for liquids. See *pH* ⟨791⟩ for guidance with pH measurement and instrument calibration. The pH of media should be in a range of ±0.2 of the value indicated by the manufacturer, unless a wider range is acceptable by the validated method.

Prepared media should be checked by appropriate inspection of plates and tubes for the following:

- Cracked containers or lids
- Unequal filling of containers
- Dehydration resulting in cracks or dimpled surfaces on solid medium
- Hemolysis
- Excessive darkening or color change
- Crystal formation from possible freezing
- Excessive number of bubbles
- Microbial contamination
- Status of redox indicators (if appropriate)
- Lot number and expiration date checked and recorded
- Sterility of the media
- Cleanliness of plates (lid should not stick to dish)

Media Storage

It is prudent to consider how the manufacturer or supplier transports and stores media before distribution to the end user. Manufacturers of media should use transport and storage conditions that minimize the loss of moisture, control the temperature, prevent microbial contamination, and provide mechanical protection to the prepared media.

Media should be labeled properly with batch or lot numbers, preparation and expiration dates, and media identification. Media should be stored according to the manufacturer's instructions. Media prepared in house should be stored under validated conditions. Do not store agar at or below 0°, as freezing could damage the gel structure. Protect stored media from exposure to light and excessive temperature. Before prolonged storage, agar plates should be placed into a sealed package or container to retard moisture loss.

Remelting of an original container of solid media should be performed only once to avoid media whose quality is compromised by overheating or potential contamination. It is recommended that remelting be performed in a heated water bath or by using free-flowing steam. The use of microwave ovens and heating plates is common, but care should be taken to avoid damaging media by overheating and to avoid the potential injury to laboratory personnel from glass breakage and burns. The molten agar medium should be held in a monitored water bath at a temperature of 45° to 50° for not more than 8 hours. Caution should be taken when pouring the media from a container immersed in a water bath to prevent water from the bath commingling with the poured sterile media. Wiping the exterior of the container dry before pouring may be advisable.

Disposal of used cultured media (as well as expired media) should follow local biological hazard safety procedures.

Quality Control Testing

Although growth media can be prepared in a laboratory from individual components, many laboratories, for ease of use, use dehydrated media or purchase commercially prepared media in plastic plates or glass containers. Manufacturers of media attempt to standardize raw materials from biological sources, but must constantly deal with unavoidable differences in raw materials obtained from natural sources, and therefore, lot-to-lot variability of media must be considered. In addition, the performance of media prepared in a laboratory or by a manufacturer is highly dependent on preparation and storage conditions. Improper media preparation can cause unsatisfactory conditions for microbial growth or recovery and unreliable results.

Therefore, quality control tests should be performed on all prepared media, including media associated with swabs or media in strips and other nontraditional formats. Tests routinely performed on in-house prepared media should include pH, growth promotion, inhibition, and indicative properties (as appropriate), and periodic stability checks to confirm the expiration dating.

When in-house prepared microbiological media are properly prepared and sterilized using a validated method, the growth-promotion testing may be limited to each incoming lot of dehydrated media, unless otherwise instructed by the relevant compendial method. If the media preparation procedure was not validated, then every batch of media should be subjected to growth-promotion testing. Test organisms may be selected from the appropriate compendial test chapter. In addition, microorganisms used in growth-promotion testing may be based on the manufacturer's recommendation for a particular medium, or may include representative environmental isolates (but these latter are not to be construed as compendial requirements).

Expiration dates on media should have supporting growth-promotion testing to indicate that the performance of the media still meets acceptance criteria up to and including the expiration date. The length of shelf life of a batch of media will depend on the stability of the ingredients and formulation under specified conditions, as well as the type of container and closure.

When a batch of media does not meet the requirements of growth-promotion testing, an investigation should be initiated to identify the cause. This investigation should include a corrective action plan to prevent the recurrence of the problem. Any batch of media that fails growth-promotion testing is unsuitable for use. [NOTE—Failed growth-promotion test results may not be used to negate positive test results.]

Some reagents are used for diagnostic purposes to help support identification of microbial organisms, e.g., Gram stain and oxidase test reagents. These may have attributes that can be quality control tested similar to microbiological media. Select the correct quality control standard microorganisms, following the manufacturer's instructions, and perform the testing before unknown sample diagnostic testing. All relevant diagnostic reagents should be subjected to incoming quality confirmation before use.

Special care should be taken with media that is used in sterility tests (see *Sterility Tests* ⟨71⟩ for requirements) and in environmental monitoring studies. Media used for environmental monitoring of critical areas should preferably be double-wrapped and terminally sterilized. If terminal sterilization is not performed, media should be subjected to 100% pre-incubation and inspection before use within a critical area. [NOTE—Growth-promotion testing for this media must be performed after the pre-incubation stage.] This will prevent extraneous contamination from being carried into controlled environments and will prevent false-positive results. A raised agar level for surface contact plates should be verified.

MAINTENANCE OF MICROBIOLOGICAL CULTURES

Biological specimens can be the most delicate standards to handle because their viability and characteristics are dependent on adequate handling and storage. Standardizing the handling and storage of cultures by the user laboratory should be done in a way that will minimize the opportunity for contamination or alteration of growth characteristics. The careful and consistent treatment of stock cultures is critically important to the consistency of microbiological test results. Cultures for use in compendial tests should be acquired from a national culture collection or a qualified secondary supplier. They can be acquired frozen, freeze-dried, on slants, or in ready-to-use forms. Confirmation of the purity of the culture and the identity of the culture should be performed before its use in quality control testing. Ready-to-use cultures should be subjected to incoming testing for purity and identity before use. The confirmation of identity for commonly used laboratory strains should ideally be done at the level of genus and species.

Preparation and resuscitation of cultures should follow the instructions of the supplier or a validated, established method. The "Seed-Lot" technique is recommended for storage of stock cultures.

The original sample from the national culture collection or a qualified secondary supplier is resuscitated and grown in an appropriate medium. Aliquots of this stock culture (the first transfer or passage) are suspended in a cryoprotective medium, transferred to vials, and frozen at −30° or below, until use. If stored at −70°, or in lyophilized form, strains may be kept indefinitely. These frozen stocks can then be used to inoculate monthly or weekly working cultures. Once opened, do not refreeze unused cell suspensions after culturing a working suspension. The unused portion should be discarded to minimize the risk of loss of viability and contamination of the stock.

The number of transfers of working control cultures should be tracked to prevent excessive subculturing that increases the risk of phenotypic alteration or mutation. The number of transfers allowable for specific compendial tests may be specified in that test. One passage is defined as the transfer of organisms from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a transfer/passage.

LABORATORY EQUIPMENT

Most equipment (incubators, water baths, and autoclaves) is subject to standard validation practices of incoming qualification, operational qualification, and performance qualification. Additionally, periodic calibration (generally annually) is commonly required. New equipment, critical to the operation of the laboratory, should be qualified according to a protocol approved by the quality assurance unit (QAU). In addition, regular cleaning and sanitization of equipment such as incubators, refrigerators, and water baths should be performed to minimize the potential for contamination in the laboratory. Door seals of incubators and refrigerators should be cleaned and checked for state of repair.

Instruments (pH meters and spectrophotometers) used in a microbiology laboratory should be calibrated on a regular schedule and tested to verify performance on a routine basis. The frequency of calibration and performance verification will vary based on the type of instrument and the importance of that equipment to the generation of data in the laboratory.

Equipment that is difficult to sanitize (such as refrigerators and incubators) should be dedicated to aseptic operations (such as storage of media for testing and incubation of sterility test samples) and live culture operations to minimize the potential for inadvertent contamination of the tests.

Autoclaves are central to the operation of the laboratory and must have proper validation in place to demonstrate adequate sterilization for a variety of operations. Autoclave resources must be available (and validated) to sterilize waste media (if performed in that laboratory) as well as the media prepared in that laboratory. The choice of one or several autoclaves is not driven by a need to separate aseptic and live operations (everything in the properly maintained autoclave is sterile after the cycle) but rather driven by resource considerations (see below).

LABORATORY LAYOUT AND OPERATIONS

Laboratory layout and design should carefully consider the requirements of good microbiological practices and laboratory safety. It is essential that cross-contamination of microbial cultures be minimized to the greatest extent possible, and it is also important that microbiological samples be handled in an environment that makes contamination highly unlikely.

In general, a laboratory should be divided into clean or aseptic areas and live culture areas. Areas in which environmental or sterile product samples are handled and incubated should be maintained completely free of live cultures, if possible. If complete separation of live and clean culture zones cannot be accomplished, then other barriers and aseptic practices should be employed to reduce the likelihood of accidental contamination. These barriers include protective clothing, sanitization and disinfection procedures, and biological safety cabinets designated for clean or aseptic operations only. Procedures for handling spills or mishaps with live cultures should be in place, and all relevant technical personnel should be trained regarding these methods.

Some samples will demonstrate microbial growth and require further laboratory analysis to identify the contaminants. When growth is detected, the sample should be taken from the clean section of the laboratory to the live culture section without undue delay. Subculturing, staining, microbial identification, or other investigational operations should be undertaken in the live culture section of the laboratory. If possible, any sample found to contain growing colonies should not be opened in the clean zone of the laboratory. Careful segregation of contaminated samples and materials will reduce false-positive results.

Staff engaged in sampling activities should not enter or work in the live culture handling section of a laboratory unless special precautions are taken, including wearing protective clothing and gloves and careful sanitizing of hands upon exiting. Ideally, staff assigned to sampling activities, particularly those in support of aseptic processing, should not work in the vicinity of live culture laboratory operations.

It is important to consider that microbial contamination of samples, which leads to false-positive results, is always possible unless careful aseptic precautions are taken. Facilities should be designed so that raw material and excipient sampling can be done under controlled conditions, including proper gowning and sterilized sampling equipment. It may not always be possible to sample utility systems, such as water systems, under full aseptic conditions; however, it should be noted that when samples are not taken aseptically, their reliability is inevitably compromised.

Environmental sampling methods should require minimal aseptic handling in loading and unloading sampling instruments. Whenever possible, sampling equipment should be loaded with its microbiological recovery media in the environment that is to be sampled.

All testing in laboratories used for critical testing procedures, such as sterility testing of final dosage forms, bulk product, seed cultures for biological production, or cell cultures used in biological production, should be performed under controlled conditions. Isolator technology is also appropriate for critical, sterile microbiological testing. Isolators have been shown to have lower levels of environmental contamination than manned clean rooms, and therefore, are generally less likely to produce false-positive results. Proper validation of isolators is critical both to ensure environmental integrity and to prevent the possibility of false-negative results as a result of chemical disinfection of materials brought into or used within isolators (see *Sterility Testing—Validation of Isolator Systems* ⟨1208⟩).

SAMPLE HANDLING

Viable microorganisms in most microbiology samples, particularly water, environmental monitoring and bioburden samples, are sensitive to handling and storage conditions. Critical parameters in these conditions include product (or sample) composition, container composition, time of storage, and temperature of storage. Therefore, it is important to minimize the amount of time between the sampling event and the initiation of testing and to control, as much as possible, the conditions of storage. If the sample is to be transported to a distant location for testing, then the conditions of transport (time, temperature, etc.) should be qualified as suitable for that test and sample. Guidance for water testing in this regard can be found in *Water for Pharmaceutical Purposes* ⟨1231⟩. Product mixing before sampling may need to be evaluated and applied in order to ensure microbial dispersement and representation in the sample aliquot.

All microbiological samples should be taken using aseptic techniques, including those taken in support of nonsterile products. If possible, all microbiological samples should be taken under full aseptic conditions in specialized sampling areas. The areas should be as close to the point of use as possible to minimize contamination during transit.

Samples submitted to the microbiology laboratory should be accompanied by documentation detailing source of the sample, date the sample was taken, date of sample submission, person or department responsible for the submission, and any

potentially hazardous materials associated with the sample. The testing department should acknowledge receipt of the sample and reconcile the identity and number of samples as part of this sample documentation.

MICROBIOLOGICAL MEDIA INCUBATION TIMES

Incubation times for microbiological tests of less than 3 days' duration should be expressed in hours: e.g., "Incubate at 30° to 35° for 18 to 72 hours". Tests longer than 72 hours' duration should be expressed in days: e.g., "Incubate at 30° to 35° for 3 to 5 days". For incubation times expressed in hours, incubate for the minimum specified time, and exercise good microbiological judgment when exceeding the incubation time. For incubation times expressed in days, incubations started in the morning or afternoon should generally be concluded at that same time of day.

TRAINING OF PERSONNEL

Each person engaged in each phase of pharmaceutical manufacture should have the education, training, and experience to do his or her job. The demands of microbiological testing require that the core educational background of the staff, supervisors, and managers be in microbiology or a closely related biological science. They should be assigned responsibilities in keeping with their level of skill and experience.

A coherent system of standard operating procedures (SOPs) is necessary to run the microbiology laboratory. These procedures serve two purposes in a training program. Firstly, these SOPs describe the methodology that the microbiologist will follow to obtain accurate and reproducible results, and so serve as the basis for training. Secondly, by tracking the procedures in which a particular microbiologist has demonstrated proficiency, the procedure number or title also serves to identify what training the microbiologist has received specific to his or her job function.

Training curricula should be established for each laboratory staff member specific to his or her job function. He or she should not independently conduct a microbial test until qualified to run the test. Training records should be current, documenting the microbiologist's training in the current revision to the particular SOP.

Periodic performance assessment is a wise investment in data quality. This performance testing should provide evidence of competency in core activities of the microbiology laboratory such as hygiene, plating, aseptic technique, documentation, and others as suggested by the microbiologist's job function.

Microbiologists with supervisory or managerial responsibilities should have appropriate education and in-house training in supervisory skills, laboratory safety, scheduling, budgeting, investigational skills, technical report writing, relevant SOPs, and other critical aspects of the company's processes as suggested in their role of directing a laboratory function.

Competency may be demonstrated by specific course work, relevant experience, and routinely engaging in relevant continuing education. Achieving certification through an accredited body is also a desirable credential. Further, it is expected that laboratory supervisors and managers have a demonstrated level of competence in microbiology at least as high as those they supervise. Expertise in microbiology can be achieved by a variety of routes in addition to academic course work and accreditation. Each company is expected to evaluate the credentials of those responsible for designing, implementing, and operating the microbiology program. Companies can thus ensure that those responsible for the program understand the basic principles of microbiology, can interpret guidelines and regulations based on good science, and have access to individuals with theoretical and practical knowledge in microbiology to provide assistance in areas in which the persons responsible for the program may not have adequate knowledge and understanding. It should be noted that microbiology is a scientifically based discipline that deals with biological principles substantially different from those of analytical chemistry and engineering disciplines. Many times it is difficult for individuals without specific microbiological training to make the transition.

LABORATORY RESOURCES

The laboratory management is responsible for ensuring that the laboratory has sufficient resources to meet the existing testing requirements. This requires some proficiency in budget management and in determining appropriate measures of laboratory performance. A measure of laboratory performance is the number of investigations performed on tests conducted by the laboratory, but this measure alone is not sufficient. In addition to tracking investigations, the period of time between sample submission and initiation of testing should be tracked, as well as the period of time between end of test and report release (or test closure). Significant delays in these measures are also indications of an under-resourced laboratory staff.

The laboratory management should have sufficient budget to meet testing requirements. Particular measures of budgetary requirements will be specific to the given laboratory, but budgetary considerations related directly to the need of the laboratory for sufficient resources must be addressed to ensure reliable testing results.

DOCUMENTATION

Documentation should be sufficient to demonstrate that the testing was performed in a laboratory and by methods that were under control. This includes, but is not limited to, documentation of the following:

- Microbiologist training and verification of proficiency

- Equipment validation, calibration, and maintenance
- Equipment performance during test (e.g., 24-hour/7-day chart recorders)
- Media preparation, sterility checks, and growth-promotion and selectivity capabilities
- Media inventory and control testing
- Critical aspects of test conducted as specified by a procedure
- Data and calculations verification
- Reports reviewed by QAU or a qualified responsible manager
- Investigation of data deviations (when required)

MAINTENANCE OF LABORATORY RECORDS

Proper recording of data and studies is critical to the success of the microbiology laboratory. The over-riding principle is that the test should be performed as written in the SOP, the SOP should be written to reflect how the test is actually performed, and the laboratory notebook should provide a record of all critical details needed to reconstruct the details of the testing and confirm the integrity of the data. At a minimum, the laboratory write-up should include the following:

- Date
- Material tested
- Microbiologist's name
- Procedure number
- Document test results
- Deviations (if any)
- Documented parameters (equipment used, microbial stock cultures used, media lots used)
- Management/Second review signature

Every critical piece of equipment should be noted in the write-up, and all should be on a calibration schedule documented by SOP and maintenance records. Where appropriate, logbooks or forms should be available and supportive of the laboratory notebook records. Equipment temperatures (water baths, incubators, autoclaves) should be recorded and traceable.

The governing SOP and revision should be clearly noted in the write-up. Changes in the data should be crossed off with a single line and initialed. Original data should not be erased or covered over.

Test results should include the original plate counts, allowing a reviewer to recreate the calculations used to derive the final test results. Methods for data analysis should be detailed in cited SOPs. If charts or graphs are incorporated into laboratory notebooks, they should be secured with clear tape and should not be obstructing any data on the page. The chart or graph should be signed by the person adding the document, with the signature overlapping the chart and the notebook page. Lab notebooks should include page numbers, a table of contents for reference, and an intact timeline of use.

All laboratory records should be archived and protected against catastrophic loss. A formal record retention and retrieval program should be in place.

INTERPRETATION OF ASSAY RESULTS

Analytical microbiological assay results can be difficult to interpret for several important reasons: (1) Microorganisms are ubiquitous in nature, and common environmental contaminants—particularly organisms associated with humans—predominate in many types of microbiological analysis; (2) the analyst has the potential to introduce contaminating organisms during sample handling or processing in the laboratory; (3) microorganisms may not be homogeneously distributed within a sample or an environment; and (4) microbiological assays are subject to considerable variability of outcome. Therefore, apparent differences from an expected outcome may not be significant.

Because of these characteristics of microbiological analysis, laboratory studies should be conducted with the utmost care to avoid exogenous contamination as previously discussed in this chapter. Equally important, results must be interpreted from a broad microbiological perspective, considering not only the nature of the putative contaminant, but the likelihood of that organism(s) surviving in the pharmaceutical ingredient, excipient, or environment under test. In addition, the growth characteristics of the microorganism should be considered (especially in questions of the growth of filamentous fungi in liquid media).

When results are observed that do not conform to a compendial monograph or other established acceptance criteria, an investigation into the microbial data deviation (MDD) is required. There are generally two distinct reasons for the observation of microbial contamination that does not comply with a target or requirement: There may be either a laboratory error or laboratory environmental conditions that produced an invalid result, or the product contains a level of contamination or specific types of contaminants outside established levels or limits. In either case, laboratory management and, in most cases, the Quality Unit should be notified immediately.

A full and comprehensive evaluation of the laboratory situation surrounding the result should be undertaken. All microbiological conditions or factors that could bring about the observed condition should be fully considered, including the magnitude of the excursion compared to established limits or levels. In addition, an estimate of the variability of the assay may be required in order to determine whether the finding is significant.

The laboratory environment, the protective conditions in place for sampling, historical findings concerning the material under test, and the nature of the material, particularly with regard to microbial survival or proliferation in contact with the mate-

General Chapters

rial, should be considered in the investigation. In addition, interviews with the laboratory analyst(s) may provide information regarding the actual conduct of the assay that can be valuable in determining the reliability of the result and in determining an appropriate course of action. If laboratory operations are identified as the cause of the nonconforming test outcome, then a corrective action plan should be developed to address the problem(s). Following the approval and implementation of the corrective action plan, the situation should be carefully monitored and the adequacy of the corrective action determined.

If assay results are invalidated on the basis of the discovery of an attributable error, this action must be documented. Laboratories also should have approved procedures for confirmatory testing (retesting), and if necessary, resampling where specific regulatory or compendial guidance does not govern the conduct of an assay investigation.

〈1118〉 MONITORING DEVICES—TIME, TEMPERATURE, AND HUMIDITY

INTRODUCTION

This chapter provides background information about the science and technology of temperature and humidity monitoring over time. It describes the available technologies and performance characteristics, and provides recommendations for qualifying performance. The shelf life of a drug product is a function of the temperature and humidity conditions during storage and transportation, as well as the drug product's chemical and physical properties. For this reason, the ability to monitor those conditions is important in the shipping and storage of temperature- and humidity-sensitive drug products. This chapter focuses strictly on supply chain temperature- and humidity-monitoring devices, both electronic and chemical.

The storage and distribution temperatures may be different if justified by appropriate stability studies and as indicated in the labeling. The effects of humidity are typically observed over longer time periods of exposure than are temperature effects due to the barrier to moisture ingress presented by the primary and secondary drug product packaging.

The devices described in this chapter are those most commonly used to monitor the controlled storage and established distribution of drug products following Good Distribution Practices (GDP).[1] The chapter does not address measurement of temperature at extremes, which are temperatures above those that drugs are reasonably expected to experience in the supply chain. The types of devices described are already used in the worldwide distribution of pharmaceuticals and by other similar industries that require temperature control in distribution (for example, the perishable food, blood component, and medical device industries). Devices also may be attached to individual items for the end user (for example, vaccine vials in the World Health Organization (WHO)/UNICEF global immunization program).[2] Appropriate recycling practices should be followed for all devices as required by local regulations.

TEMPERATURE-MEASUREMENT DEVICES

Alcohol or Mercury Thermometers

These devices are based on the change in volume of a liquid as a function of temperature. Both types of thermometers can be designed to indicate the maximum and minimum temperatures (see *General Notices, 6.80.30. Temperature Reading Devices* for more information). Historically, these types of thermometers are used in a laboratory setting or in a pharmacy, rather than during supply chain monitoring. Alcohol thermometers can have a precision as good as 0.01°, but they must be quite large to measure temperatures in ranges of more than a few degrees.

Mercury thermometers are typically used in the ranges from 0° to 50° with a precision of about 0.1°. Some local regulations apply to mercury-based thermometers, and many states and local agencies have legislated or developed collection or exchange programs for mercury-containing devices. The U.S. Environmental Protection Agency also issues regulations requiring industry to reduce mercury releases to air and water, and to properly treat and dispose of mercury wastes to avoid potential health hazards.[3] Globally, Health Care Without Harm and WHO are co-leading a Health Care Initiative Products Partnership to reduce demand for mercury-containing devices by at least 70% by 2017 and to shift production to accurate, affordable, and safer nonmercury alternatives.[4]

Both alcohol and mercury thermometers are more fragile than other temperature-measuring devices described in this chapter.

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[1] PDA Technical Report 52 (TR 52) Guidance for Good Distribution Practices (GDPs) For the Pharmaceutical Supply Chain.
[2] World Health Organization (WHO)-UNICEF Policy Statement on the Implementation of Vaccine Vial Monitors: The Role of Vaccine Vial Monitors in Improving Access to Immunization, http://whqlibdoc.who.int/hq/2007/WHO_IVB_07.04_eng.pdf.
[3] http://www.epa.gov/mercury/.
[4] http://www.noharm.org/.

Infrared Devices

This device is used for measuring the infrared (IR) radiant heat from the article whose temperature is being determined, and the IR reading varies as a function of the object's temperature. The advantage of this type of device is that the article may be at some distance from the IR sensor. IR devices may give inaccurate higher or lower temperature readings because of the surface characteristics of packages (e.g., black vs. white surfaces), and they also have the potential for operator error because of incorrect use of the IR reader (improper angle).

Resistance Temperature Detectors

The resistance temperature detector (RTD) is based on the change in electrical resistance of a material as a function of temperature. The precision and accuracy of an RTD depend on the quality of the electronics used to measure the resistance. Although RTDs are among the most stable and accurate temperature sensors, their accuracy may change with the age and temperature of the device because its electronic components are affected by age and use. Although all temperature-measurement devices should be placed on an appropriate calibration program as recommended by the manufacturer or user of the device, this calibration is particularly important for RTDs.

Solid-State Devices

Solid-state devices are based on the effect of temperature on either an integrated circuit (see *Thermistors*) or a micromechanical or microelectrical system. These devices are commonly referred to as data loggers, and can attain high precision and have the advantage of producing a digital output.

Thermistors

A thermistor is a semiconductor device whose resistance varies with temperature. Thermistors are able to detect very small changes in temperature and are accurate over a broad range of temperatures.

Thermocouples

Thermocouples are based on the change in the junction potential of two dissimilar metals as a function of temperature. Many metal pairs can be used, and each pair provides a unique range, accuracy, and precision. Precision and accuracy depend on the quality of the electronics used to measure the voltage across both metals and the type of temperature reference used.

Thermomechanical Devices

Thermomechanical devices are based on the change in length of a solid material as a function of temperature. An example of such a device is a mechanical spring, which expands or contracts as a function of temperature, thus opening and closing an electrical circuit or moving a chart pen. Typical examples are chart recorders used in cold rooms.

ELECTRONIC TIME–TEMPERATURE HISTORY RECORDERS

These recorders use one of the electronic temperature-measurement technologies described above and create a record of the temperature history experienced.

Electronic Time–Temperature Indicators

Electronic time–temperature indicators (TTIs) can be designed to alarm after a cold excursion, heat excursion, or after multiple temperature excursions and can provide a visual alarm by a colored light or LCD. The alarm(s) are generally programmable and can display conditions such as date, time, temperature, and duration of the alarm. A certificate of calibration is issued for individual units or lots. Multiple-use devices should have a calibration schedule, but single-use devices can rely on manufacturers' certificates of calibration.

Electronic Temperature-Data Loggers

Electronic temperature-data loggers are recorders that monitor the temperature at programmable intervals and save the temperature history to a peripheral system, such as a personal computer. In addition, data loggers can record humidity using sensors described below. Electronic recorders monitor and save temperature values representative of the cumulative temperature history over a period of time and therefore have the advantage of being able to calculate the mean kinetic temperature

(MKT)[5] based on the measurements. Data loggers equipped with transmitting devices (hardwired or radio transmission) can be used to monitor the temperature and humidity of a product while in transit and can download the recorded data when the data loggers arrive at a destination. Data loggers are increasingly required by worldwide ministries of health as part of a standard quality system for GDPs. Based on their communication capabilities, data loggers can be grouped into several different categories.

Radio-Frequency Data Loggers

In addition to data loggers that require a hardwired connection to a base unit or a computer, in recent years companies have adapted wireless (radio-frequency or RF-enabled) temperature and humidity recorders. The effects of radio-frequency identification (RFID) use on biologics have been studied on a variety of blood, blood components, monoclonal antibodies, and vaccines, and have demonstrated no effect.[6] These loggers are integrated with chips capable of wireless RF communications that constitute the RFID sensor tags. The RFID chip inside the tag can be either active, which requires battery power for operation, or passive, which requires a nonzero RF field created by the RFID interrogator host unit (commonly called the reader) in the vicinity of the tag. RFID-enabled sensor tags (temperature and/or humidity) have the added capability of conveying recorded temperature history wirelessly to a host computer or database for seamless downstream processing. Multiple passive and active standards exist to control the communication between the tag and the host unit, including ISO-18000-6C,[7] ISO-18000-7,[8] ZigBee,[9] IEEE 802.11,[10] and many proprietary standards.

When choosing between active and passive technologies, one needs to know that active technologies typically have extended communication range and memory capabilities at the expense of a higher price. Currently, reading ranges extend to 100 m and with repeaters longer distances can be achieved. Whether the communications circuitry is passive or active, these RF loggers still are electronic temperature recorders, which means their sensor circuitry uses external power from batteries or other sources.

A completely passive wireless RFID tag with an antenna capable of functioning as a sensor has been developed. The tag uses resonant antenna structures of RFID tags that are coated with specific sensor films. The passive wireless RFID tags act like analog sensors that, when interrogated by a wireless reader, show the instantaneous temperature. The film changes the antenna's reflection characteristics based on the monitored environmental variable (such as temperature and/or humidity), which then is decoded by the reader. The sensor film can be designed to work with different variables such as temperature, humidity, and various gas and chemical vapors. Although they lack some of the memory functionality included in electronic recorders, passive wireless sensors are relatively cost effective compared to data loggers and can be considered for item-level applications.

CHEMICAL TEMPERATURE INDICATORS

Chemical temperature indicators are relatively cost effective compared to electronic data loggers and can be considered for item-level applications There are two basic types of chemical temperature indicators: 1) a threshold indicator that responds at a specific temperature and 2) a TTI that responds to cumulative heat exposure.

Chemical Temperature Threshold Indicators

These indicators, sometimes referred to as critical temperature indicators, are based on a phase change or chemical reaction that occurs as a function of temperature. Examples include liquid crystals, waxes, polymers, and lacquers that change phase, and thereby their appearance, as a function of temperature. Chemical temperature threshold indicators are reversible or irreversible and are suitable for high or low temperatures. Temperature threshold indicators do not include any specified time delay to show a response and typically are single-use devices. These indicators provide a signal only when exposed to temperatures higher than (ascending indicator) or lower than (descending indicator) a predetermined threshold temperature.

Ascending-Temperature Threshold Indicators—Ascending-temperature threshold indicators are supplied as self-adhesive labels or cards and are normally composed of a heat-fusible compound that melts at the critical temperature. Melting of the compound gives rise to a color change or color development. Other types are provided as inks, lacquers, pellets, or crayons. Ascending-temperature threshold indicators are available from 0° to more than 200° and as many as 10 temperatures on a single unit. Some ascending-temperature threshold indicators used to monitor frozen or refrigerated temperatures require an activation step (such as a pull tab or a reservoir that is ruptured by pressure).

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[5] The Use of Mean Kinetic Temperature (MKT) in the Handling, Storage, and Distribution of Temperature Sensitive Molecules. R. H. Seevers, J. Hofer, P. Haber, D. A. Ulrich, R. Bishara. Pharmaceutical Outsourcing, May/June, 30–37, 2009.

[6] Effects of Radio Frequency Identification-Related Radiation on In Vitro Biologics. I. Uysal, C. Hohberger, R. S. Rasmussen, D. A. Ulrich, J. P. Emond, A. Gutierrez. PDA Journal of Pharmaceutical Science and Technology, Vol. 66(4), July/Aug, 333–345, 2012.

[7] ISO/IEC 18000-6:2010–ISO/IEC 18000-6:2010—Information technology–
Radio frequency identification for item management–Part 6: Parameters for air interface communications at 860 MHz to 960 MHz.

[8] ISO/IEC 18000-7:2009—Information technology–Radio frequency identification for item management–Part 7: Parameters for active air interface communications at 433 MHz.

[9] IEEE 802.15.4—Wireless Medium Access Control (MAC) and Physical Layer (PHY) Specifications for Low-Rate Wireless Personal Area Networks (LR-WPANs), 2003.

[10] IEEE 802.11—Wireless Local Area Networks (LANs), 2007.

Descending-Temperature Threshold Indicators—Descending-temperature threshold indicators show a response when exposed to temperatures below a threshold. These indicators do not include a specific time delay to show a response, and the response is typically caused by the time required for solidification of a liquid at the threshold temperature. Solidification of a liquid causes a visual change in the indicator. Examples include: 1) the expansion of the liquid to crack an ampule and release a colorant, 2) contraction of the liquid to mix components to develop color, or 3) aggregation of colloidal particles to change color.

Chemical Time–Temperature Indicators

These indicators, sometimes referred to as time–temperature integrators (TTIs), include systems in which a reaction rate or diffusion process is used to estimate a temperature equivalent integrated over time. Thus, TTIs provide a measure of accumulated heat rather than instantaneous temperature such as a spike or critical threshold (discussed above). The reactions generally are irreversible—once a color change, color development, or diffusion process has taken place, exposure to low temperatures will not restore the indicator to its original state, but lower temperatures (refrigeration) will slow the color change. The accuracy and precision of these indicators depend, to some extent, on human interpretation. Some versions of chemical indicators have been prepared in a bar code format and can be read with bar code readers. Other developments include reading a chemical indicator with an imaging device such as a camera in a smart phone.

TTIs do not directly reflect the status of the drugs to which they are attached. In actual practice, the characteristics for degradation of a particular drug are known from accelerated and real-time stability studies that follow internationally accepted guidelines such as PDA TR 53[11] to guide selection of a suitable TTI.[12] The activation energy of the TTI is not required to exactly match the activation energy of the degradation of the drug being monitored, and the latter, in fact, may not be known precisely. Therefore, the TTI should be chosen to provide an early warning if the drug is exposed to an excessive heat load before the expiration date.

An important characteristic of chemical TTIs is the precision with which the end point can be determined. For TTIs that change color, a reference color normally brackets the active portion of the TTI to show the end point color, which simplifies TTI interpretation. Accuracy can vary widely with the control and quality of the manufacturing process. Some TTIs are manufactured by procedures that comply with Quality System Regulations for Medical Devices. As discussed below in *Calibration of Temperature- and Humidity-Monitoring Devices*, it is not possible to calibrate any individual single-use device because the test is, by the nature of the TTI, necessarily destructive. This is analogous to any pharmaceutical product because each dose cannot be calibrated or validated, but validated processes should be used in the manufacturing process.

The two types of TTIs are partial-history indicators and full-history indicators. Partial-history TTIs provide a time- and temperature-dependent response when the temperature exceeds a predetermined value. A partial-history indicator normally is composed of a dyed, heat-fusible compound that diffuses along a porous strip or wick when the temperature exceeds the melting point of the compound. The diffusion process of the compound down the wick is temperature dependent, and therefore the partial-history TTI provides a time and temperature response above the melting point of the compound. Migration of the compound down the wick stops when the indicator is moved to a lower temperature at which point the compound solidifies. These TTIs normally have one or more viewing windows to monitor the length the dyed compound has traveled along the wick. Some indicators are activated by removing a barrier film that separates the dyed compound from the wick or rupturing a reservoir that contains the dyed compound. Other indicators do not require activation and must be stored below the melting point of the compound before use. These partial-history TTIs can have durations (service life) anywhere from several hours to several years. Full-history TTIs provide a continuous response to temperature. They change color or physical appearance as a result of exposure to time, and the rate of change increases with temperature so they are sensitive to cumulative heat exposure. Full-history TTIs are responsive to MKT (Ref 1079) and typically are single use, irreversible, and disposable because once the color changes it will not revert to the original color.

Chemical–Physical Time–Temperature Indicators

This type of TTI is based on a temperature-dependent diffusion or chemical reaction process. It consists of a pressure-sensitive tape device that is composed of an indicator tape and an activator tape. In one example, the indicator tape contains a dye precursor dispersed in a polymer carrier. The activator is incorporated into an adhesive on the activator tape. Laminating the activator tape over the indicator tape causes activation. A color change or readable message occurs as the activator migrates into the indicator as a function of temperature and time. Other approaches to develop color changes include the use of a pH indicator or the etching of aluminum by the activator tape.

Chemical Polymerization–Based Time–Temperature Indicators

This type of TTI uses a solid-state polymerization process in which a color develops intensity as a function of time and temperature. The color evolution is caused by the polymerization of a colorless monomer to a highly colored polymer. These TTIs

[11] PDA Technical Report 53 (TR 53) Guidance for Industry: Stability Testing to Support Distribution of New Drug Products, https://store.pda.org.
[12] ASTM F1416–96 (2008) Standard Guide for Selection of Time Temperature Indicators.

can be applied by a print process that permits direct integration into a product label or stand-alone label. Because this type of TTI does not require activation, it must be shipped from the manufacturer on dry ice or under frozen conditions and stored at temperatures according to the manufacturer's instructions, normally below −24° before use. Chemical polymerization–based TTIs can be designed to reach the end point as quickly as weeks at refrigerated temperature or as long as years at controlled room temperature.

Chemical Enzyme–Based Time–Temperature Indicators

This type of TTI uses an enzyme-catalyzed color-generating reaction that occurs as a function of time and temperature. The color change is caused by an enzyme reacting with a substrate, accompanied by a change in pH. The enzyme and substrate are in separate solutions in adjacent compartments. Breaking the barrier between the two compartments and mixing the two solutions activates the TTI.

Chemical-Organic Pigment–Based Time–Temperature Indicators

This type of TTI uses an organic pigment that is activated by exposure to ultraviolet light to develop a dark blue starting color. A filter is then placed over the label to protect it from deliberate or accidental reactivation. The colored pigment fades over time as a function of temperature.

RELATIVE HUMIDITY MEASUREMENT TECHNOLOGIES

Relative humidity is the ratio of the partial pressure of water vapor in air to the vapor pressure of saturated air at a given temperature. In other words, the relative humidity is the amount of water vapor present, divided by the theoretical amount of moisture that could be held by that volume of air at a given temperature. Extensive tables of relative humidity data are available. Devices for measuring relative humidity are called hygrometers. Several different technologies exist for measuring relative humidity.

Sling Psychrometer

The simplest type of hygrometer is based on the temperature difference observed between two identical thermometers, one ordinary and one with a wet cloth wick over its bulb. The two thermometers are whirled at the end of a chain, and the evaporation of water from the wick cools (based on evaporative cooling) the wet-bulb thermometer. The temperature difference between the wet and dry thermometers then is compared to a table specific to that psychrometer based on dry-bulb temperature, and the relative humidity is determined.

Hair Hygrometer

This type of device is based on the fact that the length of a synthetic or human hair increases as a function of the relative humidity. This change is used to move an indicator or affect a strain gauge. A hair hygrometer can be accurate to ±3%, but it is unable to respond to rapid changes in humidity and loses accuracy at very high or very low levels of relative humidity.

Infrared Hygrometer

This type of hygrometer determines relative humidity by comparing the absorption of two different wavelengths of IR radiation through air. One wavelength is absorbed by water vapor and the other is not. This type of hygrometer can accurately measure relative humidity in large or small volumes of air. It is sensitive to rapid changes of humidity and can be integrated with an electronic data-handling system.

Dew Point Hygrometer

This type of device uses a chilled mirror to determine the dew point of an air sample. The dew point is the temperature at which water vapor in the air begins to condense; that is, the temperature at which the relative humidity is 100%. The relative humidity can be calculated from this measurement and an accurate measurement of the ambient temperature. The dew point hygrometer is the standard against which most commercially available instruments are calibrated.

Capacitive Thin-Film Hygrometer

The principle of this type of hygrometer is that the dielectric of a nonconductive polymer changes in direct proportion to the relative humidity. This change is measured as a change in capacitance. This type of hygrometer is accurate to ±3%.

Resistive Thin-Film Hygrometer

This type of hygrometer is similar to the capacitive thin-film type because it uses the effect of changing relative humidity on an electric circuit. In the resistive thin-film hygrometer the sensor is an organic polymer whose electrical resistance changes in logarithmic proportion to the relative humidity. This type of hygrometer is accurate to ±5%.

CALIBRATION OF TEMPERATURE- AND HUMIDITY-MONITORING DEVICES

Thermometers and hygrometers that are used to provide data about the temperature and humidity exposure of a product must be suitable for their intended use. Specifically, they must be appropriately calibrated. A calibration program assures the user of the monitoring device that the device has been tested before use either by the manufacturer or the user to assess the suitability for its intended use. Calibrations should be performed with appropriate frequencies to support ongoing use. Monitors used in manufacturing, storage, and transport of drug products should be properly qualified by their users to ensure that the monitors have been received and maintained in proper working order. It is acceptable to use the calibration performed by the device's manufacturer based on the certificate of calibration and expiration date.

For temperature- and humidity-monitoring devices, measurement accuracy refers to the closeness of the value obtained with a particular device and the true value of the object or environment under measurement. In practice, this is determined by comparison with a device that has been calibrated against a standard that is obtained from or is traceable to the National Institute of Standards and Technology or a comparable national metrology organization.

Any monitor takes time to respond to a change in the temperature or humidity. Measurement responsiveness typically is defined in a device's specifications for its operating range. Different data recording intervals are appropriate for different monitoring applications and should be based on supply chain length (for example, transportation via ocean may require 30-min intervals, but 15-min intervals may be suitable for air transport). Most commonly, time accuracy is expressed as a ± percentage of total duration of the recording period. For pharmaceutical applications, a ±0.5% time accuracy is adequate.

Single-use electronic and chemical indicators should follow Good Manufacturing Practices with appropriate testing controls. Electronic indicators require proper calibration. Single-use indicator performance can be qualified by the supply chain user by sampling and testing of multiple production lots. For TTIs that calculate MKT, the performance of a batch can be assessed statistically by subjecting an appropriately sized sample to elevated temperature conditions for a set period of time and observing the results. Manufacturers should adopt appropriate acceptance criteria. It is acceptable to use the release test performed by the manufacturer of the indicator (based on the certificate of calibration or the certificate of analysis and the expiration date) in lieu of calibration or qualification.

THE USE OF HISTORICAL TEMPERATURE DATA

Although historical geographic and seasonal trends may be used as a planning tool in selecting among the types of temperature- and humidity-monitoring devices, outside ambient temperatures are not necessarily reliable indicators of the temperatures experienced by different items in the distribution chain. For example, studies have reported important departures from ambient temperatures on summer days for mailboxes, trucks, and warehouses.[13] Therefore, using lane-specific temperature monitoring is beneficial when manufacturers and shippers develop an ambient profile and can be a valuable consideration for a risk-based approach to maintain product quality.[14]

A drug product's quality (identity, strength, and purity) may be notably influenced by variations in temperature and humidity over the course of its shelf life, so manufacturers should appropriately monitor those environmental conditions. Pharmaceutical manufacturers perform stability testing to carefully evaluate the effects of temperature and humidity on their products. The packaging, shelf life, and storage and transportation conditions recommended for a product are chosen based on the results of these stability studies. Temperature effects can happen rapidly; therefore, temperature monitoring should be implemented on a risk-based approach taking the product stability, distribution route, mode of transportation and potential risks that may compromise the quality of the product into account. Relative humidity effects occur over a much longer time frame; humidity monitoring can be omitted when the drug product is sufficiently protected by the primary container proven by sound stability studies. Humidity monitoring is recommended when special environmental restrictions concerning the humidity are defined for the drug product.

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[13] Okeke, C.C. Medwick, T., Bailey, L.C., and Grady L.T. Temperature and Humidity Conditions During Shipment in International Commerce, *PF* 25(2) Mar.–Apr. 1999.

[14] ISTA 7E Standard, http://www.ista.org.

⟨1119⟩ NEAR-INFRARED SPECTROSCOPY

INTRODUCTION

Near-infrared (NIR) spectroscopy is a branch of vibrational spectroscopy that shares many of the principles that apply to other spectroscopic measurements. The NIR spectral region comprises two subranges associated with detectors used in the initial development of NIR instrumentation. The short-wavelength (Herschel or silicon region) extends from approximately 780 to 1100 nm (12,821–9000 cm^{-1}); and longer wavelengths, between 1100 and 2500 nm, compose the traditional (lead sulfide) NIR region. Applications of NIR spectroscopy use spectra displayed in either wavelength or wavenumber units. As is the case with other spectroscopy measurements, interactions between NIR radiation and matter provide information that can be for both qualitative and quantitative assessment of the chemical composition of samples. In addition, qualitative and quantitative characterization of a sample's physical properties can be made because of the sample's influence on NIR spectra. Measurements can be made directly on samples in situ in addition to applications during standard sampling and testing procedures.

Applications of qualitative analysis include identification of raw material, in-process sample, or finished product. These applications often involve comparing an NIR spectrum from a sample to reference spectra and assessing similarities against acceptance criteria developed and validated for a specific application. In contrast, applications of quantitative analysis involve the development of a predictive relationship between NIR spectral attributes and sample properties. These applications typically use numerical models to quantitatively predict chemical and/or physical properties of the sample on the basis of NIR spectral attributes.

Vibrational spectroscopy in the NIR region is dominated by overtones and combinations that are much weaker than the fundamental mid-IR vibrations from which they originate. Because molar absorptivities in the NIR range are low, radiation can penetrate several millimeters into materials, including solids. Many materials, such as glass, are relatively transparent in this region. Fiber-optic technology is readily implemented in the NIR range, which allows monitoring of processes in environments that might otherwise be inaccessible.

The instrument qualification tests and acceptance criteria provided in this chapter may not be appropriate for all instrument configurations. In such cases, alternative instrument qualification and performance checks should be scientifically justified and documented. In addition, validation parameters discussed in this chapter may not be applicable for all applications of NIR spectroscopy. Validation parameters characterized for a specific NIR application should demonstrate suitability of the NIR application for its intended use.

Transmission and Reflection

The most common measurements performed in the NIR spectral range are transmission and reflection spectroscopy. Incident NIR radiation is absorbed or scattered by the sample and is measured as transmittance or reflectance, respectively. Transflection spectrometry is a hybrid of transmission and reflection wherein a reflector is placed behind the sample so that the optical path through the sample and back to the detector is doubled compared to a transmission measurement of a sample of the same thickness. Transflection is used to describe any double-pass transmission technique. The light may be reflected from a diffuse or specular (mirror) reflector placed behind the sample. This configuration can be adapted to share instrument geometry with certain reflection or fiber-optic probe systems in which the source and the detector are on the same side of the sample.

TRANSMITTANCE, T, is a measure of the decrease in radiation intensity as a function of wavelength when radiation is passed through a sample. The sample is placed in the optical beam between the source and the detector. The results of both transmission and transflection measurements are usually presented directly in terms of absorbance, i.e., $\log_{10}(1/T)$.

REFLECTANCE, R, is a measure of the ratio of the intensity of light reflected from the sample, I, to that reflected from a background or reference reflective surface, I_R. Most reflection measurements in the NIR are made of scattering samples such as powders and slurries. For such materials NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an *overtone*) or the sum of two or more different modes (a *combination band*). Nonabsorbed radiation is scattered back from the sample to the detector. NIR reflection spectra are accessed by calculating and plotting $\log(1/R)$ versus wavelength. This logarithmic form is the pseudo-absorbance of the material and is commonly called absorbance.

Factors That Affect NIR Spectra

The following list is not exhaustive, but it includes many of the major factors that affect NIR spectra.

Sample Temperature—Sample temperature influences spectra obtained from aqueous solutions and other hydrogen-bonded liquids, and a difference of a few degrees may result in significant spectral changes. Temperature may also affect spectra obtained from less polar liquids, as well as solids that contain solvents and/or water.

Moisture and Solvent—Moisture and solvent present in the sample material and analytical system may change the spectrum of the sample. Both absorption by moisture and solvent and their influence on hydrogen bonding of the APIs and excipients can change the NIR spectrum.

Sample Thickness—Sample thickness is a known source of spectral variability and must be understood and/or controlled. The sample thickness in transmission mode is typically controlled by using a fixed optical path length for the sample. In diffuse reflection mode, the sample thickness is typically controlled by using samples that are "infinitely thick" relative to the detectable penetration depth of NIR light into a solid material. Here "infinite thickness" implies that the reflection spectrum does not change if the thickness of the sample is increased.

Sample Optical Properties—In solids, both surface and bulk scattering properties of calibration standards and analytical samples must be taken into account. Surface morphology and refractive index properties affect the scattering properties of solid materials. For powder materials, particle size and bulk density influence scattering properties and the NIR spectrum.

Polymorphism—Variation in crystalline structure (polymorphism) from materials with the same chemical composition can influence NIR spectral response. Different polymorphs and amorphous forms of solid material may be distinguished from one another on the basis of their NIR spectral properties. Similarly, different crystalline hydration or solvation states of the same material can display different NIR spectral properties.

Age of Samples—Samples may exhibit changes in their chemical, physical, or optical properties over time. Care must be taken to ensure that both samples and standards used for NIR analysis are suitable for the intended application.

INSTRUMENTATION

Apparatus

All NIR measurements are based on exposing material to incident NIR light radiation and measuring the attenuation of the emerging (transmitted, scattered, or reflected) light. Several spectrophotometers are available; they are based on different operating principles—for example: filters, grating-based dispersive, acousto-optical tunable filter (AOTF), Fourier–transform NIR (FT–NIR), and liquid crystal tunable filter (LCTF). Silicon, lead sulfide, indium gallium arsenide, and deuterated triglycine sulfate are common detector materials. Conventional cuvette sample holders, fiber-optic probes, transmission dip cells, and spinning or traversing sample holders are common examples of sample interfaces for introducing the sample to the optical train of a spectrometer.

The selection of specific NIR instrumentation and sampling accessories should be based on the intended application, and particular attention should be paid to the suitability of the sampling interface for the type of sample that will be analyzed.

Near-Infrared Reference Spectra

NIR references, by providing known stable measurements to which other measurements can be compared, are used to minimize instrumental variations that would affect the measurement.

Transmittance—The measurement of transmittance requires a background reference spectrum for determining the absorption by the sample relative to the background. Suitable transmittance reference materials depend on the specific NIR application and include air, an empty cell, a solvent blank, or a reference sample.

Reflectance—The measurement of reflectance requires the measurement of a reference reflection spectrum to determine the attenuation of reflected light relative to the unattenuated incident beam. The reflectance spectrum is calculated as the ratio of the single-beam spectrum of the sample to that of the reference material. Suitable reflectance reference materials depend on the specific NIR application and include ceramic, perfluorinated polymers, gold, and other suitable materials.

Qualification of NIR Instruments

Qualification—Qualification of an NIR instrument can be divided into three elements: Installation Qualification (IQ); Operational Qualification (OQ); and Performance Qualification (PQ). For further discussion, see general information chapter *Analytical Instrument Qualification* ⟨1058⟩.

Installation Qualification—The IQ requirements help ensure that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

Operational Qualification—In operational qualification, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of operational qualification is to demonstrate that instrument performance is suitable. Because there are so many different approaches for measuring NIR spectra, operational qualification using standards with known spectral properties is recommended. Using external traceable reference standard materials does not justify omitting the instrument's internal quality control procedures. As is the case with any spectroscopic device, wavelength uncertainty, photometric linearity, and noise characteristics of NIR instruments should be qualified against target specifications for the intended application.

Performance Qualification—Performance qualification demonstrates that the NIR measurement consistently operates within target specifications defined by the user for a specific application; it is often referred to as *system suitability*. Performance quali-

fication for NIR measurements can include comparing a sample or standard spectrum to previously recorded spectra. Comparisons of spectra taken over time from identical and stable samples or reference standard materials can form the basis for evaluating the long-term stability of an NIR measurement system. The objective is to demonstrate that no abnormal wavelength shift or change in detector sensitivity has occurred during ongoing analysis.

Characterizing Instrument Performance—Specific procedures, acceptance criteria, and time intervals for characterizing NIR instrument performance depend on the instrument and intended application. Many NIR applications use previously validated models that relate NIR spectral response to a physical or chemical property of interest. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from sample spectra using previously validated NIR models.

Wavelength Uncertainty—NIR spectra from sample and/or reference standard materials can be used to demonstrate an instrument's suitable wavelength dispersion performance against target specifications. The USP Near IR System Suitability Reference Standard or the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2036 for reflectance measurement and NIST SRM 2035 for transmittance measurement can be used for wavelength verification. Suitable materials for demonstrating wavelength dispersion performance include polystyrene, mixtures of rare earth oxides, and absorption by water vapor for instruments that use an interferometer for wavelength dispersion. With appropriate justification, alternative standards may be used. Wavelength uncertainty typically is characterized from a single spectrum (collected with the same spectral resolution to obtain the standard value) using a minimum of three peaks that cover a suitable spectral range of the instrument. Typical tolerances for agreement with standard values are ±1.0 nm below 2000 nm and ±1.5 nm from 2000 nm to 2500 nm. Alternative tolerances may be used when justified for specific applications.

Photometric Linearity and Response Stability—NIR spectra from samples and/or reference standard materials with known relative transmittance or reflectance can be used to demonstrate a suitable relationship between NIR light attenuation (due to absorption) and instrument response. For reflectance measurements, commercially available reflectance standards with known reflectance properties are often used. Spectra obtained from reflection standards are subject to variability as a result of the difference between the experimental conditions under which they were factory calibrated and those under which they are subsequently put to use. Hence, the reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an "absolute" calibration for a given instrument. Provided that (1) the standards do not change chemically or physically, (2) the same reference background is also used to obtain the standard values, and (3) the instrument measures each standard under identical conditions (including precise sample positioning), the reproducibility of the photometric scale will be established over the range of standards. Subsequent measurements on the identical set of standards give information on long-term stability. Photometric linearity is typically characterized using a minimum of four reference standards in the range from 10% to 90% reflection (or transmission). NIR applications based on measuring an absorbance larger than 1.0 may require standards with reflectivity properties between 2% and 5% reflection (or transmission) for characterizing instrument performance at low reflectance. The purpose is to demonstrate a linear relationship between NIR reflectance and/or transmittance and instrument response over the scanning range of the instrument. Typical tolerances for a linear relationship are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept of a plot of the measured photometric response versus standard photometric response. Alternative tolerances may occur when justified for specific applications.

Spectroscopic Noise—NIR instrument software may include built-in procedures to automatically determine system noise and to provide a statistical report of noise or S/N over the instrument's operating range. In addition, it may be desirable to supplement such checks with measurements that do not rely directly on manufacturer-supplied procedures. Typical procedures involve measuring spectra of traceable reference materials with high and low reflectance. Tolerances for these procedures should demonstrate suitable S/N for the intended application.

HIGH-FLUX NOISE—Instrument noise is evaluated at high-light flux by measuring reflectance or transmittance of the reference standard, with the reference material (e.g., 99% reflection standard) acting as both the sample and the background reference.

LOW-FLUX NOISE—The same procedure may be used with a lower-reflectivity reference material (e.g., 10% reflectance standard) to determine system noise at reduced light flux. The source, optics, detector, and electronics make significant contributions to the noise under these conditions.

METHOD VALIDATION

Introduction

The objective of NIR method validation, as is the case with the validation of any analytical procedure, is to demonstrate that the measurement is suitable for its intended purpose. NIR spectroscopy is somewhat different from conventional analytical techniques because validation of the former generally is achieved by the assessment of chemometric parameters, but these parameters can still be related to the fundamental validation characteristics required for any analytical method.

Data pretreatment is often a vital step in the chemometric analysis of NIR spectral data. Data pretreatment can be defined as the mathematical transformation of NIR spectral data to enhance spectral features and/or remove or reduce unwanted sources of variation prior to using the spectrum. *Calibration* is the process of developing a mathematical relationship between NIR spectral response and properties of samples. Many suitable chemometric algorithms for data pretreatment and calibration exist; the selection should be based on sound scientific judgment and suitability for the intended application.

Validation Parameters

Performance characteristics that demonstrate the suitability of NIR methods are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Validation of Compendial Procedures* ⟨1225⟩. These principles should be considered typical for NIR procedures, but exceptions should be dealt with on a case-by-case basis. For qualitative NIR methods, see chapter ⟨1225⟩, *Data Elements Required for Validation, Category IV* assays. For quantitative NIR methods, see chapter ⟨1225⟩, *Data Elements Required for Validation, Category I* and *Category II* assays. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. The samples for validation should be independent of the calibration set.

Specificity—The extent of specificity testing depends on the intended application. Demonstration of specificity in NIR methods is typically accomplished by using the following approaches:

Qualitative—Identification testing is a common application of qualitative NIR spectroscopy. Identification is achieved by comparing a sample spectrum to a reference spectrum or a library of reference spectra. The specificity of the NIR identification method is demonstrated by obtaining positive identification from samples coupled with negative results from materials that should not meet criteria for positive identification. Materials to demonstrate specificity should be based on sound scientific judgment and can include materials similar in visual appearance, chemical structure, or name.

Quantitative—Quantitative applications of NIR spectroscopy typically involve establishing a mathematical relationship between NIR spectral response and a physical or chemical property of interest. Demonstrating specificity against a physical or chemical property of interest is based on interpreting both NIR spectral attributes and chemometric parameters in terms of the intended application and may include the following:

- Spectral regions in the calibration model can be correlated to a known NIR spectral response associated with the property of interest.
- Wavelengths used by regression analysis for the calibration (e.g., for multiple linear regression [MLR] models) or the loading vector for each factor (e.g., for partial least squares [PLS] or principal component regression [PCR] models) can be examined to verify relevant spectroscopic information that is used for the mathematical model.
- Variation in spectra from samples for calibration can be examined and interpreted as expected spectral observations.
- Variation in material composition and sample matrix may be shown to have no significant effect on quantification of the property of interest within the specified method range.

Linearity—Quantitative NIR methods generally attempt to demonstrate a linear relationship between NIR spectral response and the property of interest. Although demonstrating a linear response is not required for all NIR applications, the model chosen, whether linear or not, should properly represent the relationship.

Validation of linearity in NIR methods may be accomplished by examining a plot of NIR spectral response versus actual or accepted values for the property of interest. Many statistical methods are available for evaluation of the goodness of fit of the linear relationship. Other applicable statistics and graphical methods may be as appropriate.

The correlation coefficient, *r*, may not be an informative measure of linearity. The square of the (Pearson) correlation coefficient is a measure of the fraction of the data's variation that is adequately modeled by the equation. Linearity depends on the standard error of the calibration equation (and hence the reference method) and on the range of the calibration data. Thus, although values very near 1.00, such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability around the line.

Range—The specified range of an NIR method depends on the specific application. The range typically is established by confirming that the NIR method provides suitable measurement capability (accuracy and precision) when applied to samples within extreme limits of the NIR measurement. Controls must be used to ensure that results outside the validated range are not accepted. In certain circumstances, it may not be possible or desirable to extend the validated range to include sample variability outside the validated range. Extending the range of an NIR method requires demonstration of suitable measurement capability within the limits of the expanded range. Examples of situations in which only a limited sample range may be available are samples from a controlled manufacturing process and in-process samples. A limited method range does not preclude the use of an NIR method.

Accuracy—Accuracy in NIR methods is demonstrated by showing the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value. Accuracy can be determined by direct comparison between NIR validation results and actual or accepted reference values. Suitable agreement between NIR and reference values is based on required measurement capability for a specific application. The purpose is to demonstrate a linear relationship between NIR results and actual values. Accuracy can be determined by agreement between the standard error of prediction (SEP) and the standard error of the reference method for validation. The error of the reference method may be known on the basis of historical data, through validation results specific to the reference method, or by calculating the standard error of the laboratory (SEL). Suitable agreement between SEP and SEL is based on required measurement capability for a specific application.

Precision—The precision of an NIR method expresses the closeness of agreement between a series of measurements under prescribed conditions. Two levels of precision should be considered: repeatability and intermediate precision. The precision of an NIR method typically is expressed as the relative standard deviation of a series of NIR method results and should be suitable for the intended application. Demonstration of precision in NIR methods may be accomplished using the following approaches:

Repeatability—Repeatability can be demonstrated by the following:

- Statistical evaluation of a number of replicate measurements of the sample without repositioning the sample between each individual spectral acquisition, or
- Statistical evaluation of multiple NIR method results, each result from a replicate analysis of a sample subsequent to repositioning between spectral acquisitions

Intermediate Precision—Intermediate precision can be shown by the following:

- Statistical evaluation of a number of replicate NIR measurements of the same or similar samples in the *Repeatability* study by different analysts on different days.

Robustness—NIR measurement parameters selected to demonstrate robustness will vary depending on the application and the sample's interface with the NIR instrument. Critical measurement parameters associated with robustness often are identified and characterized during method development. Typical measurement parameters include the following:

- Effect of environmental conditions (e.g., temperature, humidity, and vibration)
- Effect of sample temperature
- Sample handling (e.g., probe depth, compression of material, sample depth/thickness, sample presentation)
- Influence of instrument changes (e.g., lamp change, warm-up time)

Ongoing Method Evaluation

Validated NIR methods should be subject to ongoing performance evaluation, which may include monitoring accuracy, precision, and other suitable method parameters. If performance is unacceptable, corrective action is necessary. It involves conducting an investigation to identify the cause of change in method performance and may indicate that the NIR method is not suitable for continued use. Improving the NIR method to meet measurement suitability criteria may require additional method development and documentation of validation experiments demonstrating that the improved method is suitable for the intended application. The extent of revalidation required depends on the cause of change in method performance and the nature of corrective action required in order to establish suitable method performance. Appropriate change controls should be implemented to document ongoing method improvement activities.

Revalidation of a qualitative model may be necessary as a result of the following:

- Addition of a new material to the spectral reference library
- Changes in the physical properties of the material
- Changes in the source of material supply
- Identification of previously unknown critical attribute(s) of material(s)

Revalidation of a quantitative model may be necessary as a result of the following:

- Changes in the composition of the test sample or finished product
- Changes in the manufacturing process
- Changes in the sources or grades of raw materials
- Changes in the reference analytical method
- Major changes in instrument hardware

Outliers—Sample spectra that produce an NIR response that differs from the qualitative or quantitative calibration model may produce an outlier. This does not necessarily indicate an out-of-specification result; but rather an outlier indicates that further testing of the sample may be required and is dependent on the particular NIR method. If subsequent testing of the sample by an appropriate method indicates that the property of interest is within specifications, then the sample meets its specifications. Outlier samples may be incorporated into an updated calibration model subsequent to execution and documentation of suitable validation studies.

Method Transfer

Controls and measures for demonstrating the suitability of NIR method performance following method transfer are similar to those required for any analytical procedure. Exceptions to general principles for conducting method transfer for NIR methods should be justified on a case-by-case basis. The transfer of an NIR method is often performed by using an NIR calibration model on a second instrument that is similar to the primary instrument used to develop and validate the method. When a calibration model is transferred to another instrument, procedures and criteria must be applied to demonstrate that the calibration model meets suitable measurement criteria on the second instrument. The selection of an appropriate calibration model transfer procedure should be based on sound scientific judgment.

GLOSSARY

ABSORBANCE, *A*, is represented by the equation:

$$A = -\log T = \log(1/T)$$

where *T* is the transmittance of the sample. Absorbance is also frequently given as:

$$A = \log(1/R)$$

where R is the reflectance of the sample.

BACKGROUND SPECTRUM is used for generating a sample spectrum with minimal contributions from instrument response. It is also referred to as a *reference spectrum* or *background reference*. The ratio of the sample spectrum to the background spectrum produces a transmittance or reflectance spectrum dominated by NIR spectral response associated with the sample. In reflection measurements, a highly reflective diffuse standard reference material is for the measurement of the background spectrum. For transmission measurement, the background spectrum may be measured with no sample present in the spectrometer or using a cell with the solvent blank or a cell filled with appropriate reference material.

CALIBRATION MODEL is a mathematical expression to relate the response from an analytical instrument to the properties of samples.

DIFFUSE REFLECTANCE is the ratio of the spectrum of radiated light penetrating the sample surface, interacting with the sample, passing back through the sample's surface, and reaching the detector to the background spectrum. This is the component of the overall reflectance that produces the absorption spectrum of the sample.

FIBER-OPTIC PROBES consist of two components: optical fibers that may vary in length and in the number of fibers and a terminus, which contains specially designed optics for examination of the sample matrix.

INSTALLATION QUALIFICATION is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, is properly installed in the selected environment, and that this environment is suitable for the instrument's intended purpose.

INSTRUMENT BANDWIDTH OR RESOLUTION is a measure of the ability of a spectrometer to separate radiation of similar wavelengths.

MULTIPLE LINEAR REGRESSION is a calibration algorithm to relate the response from an analytical instrument to the properties of samples. The distinguishing feature of this algorithm is the use of a limited number of independent variables. Linear-least-squares calculations are performed to establish a relationship between these independent variables and the properties of the samples.

OPERATIONAL QUALIFICATION is the process by which it is demonstrated and documented that an instrument performs according to specifications and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, or major repair.

OVERALL REFLECTANCE is the sum of diffuse and specular reflectance.

PARTIAL LEAST SQUARES (PLS) is a calibration algorithm to relate instrument responses to the properties of samples. The distinguishing feature of this algorithm is that data concerning the properties of the samples for calibration are used in the calculation of the factors to describe instrument responses.

PERFORMANCE QUALIFICATION is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Performance qualification may employ the same or different standards for different performance characteristics.

PHOTOMETRIC LINEARITY, also referred to as *photometric verification*, is the process of verifying the response of the photometric scale of an instrument.

PRINCIPAL COMPONENT REGRESSION (PCR) is a calibration algorithm to relate the response from an analytical instrument to the properties of samples. This algorithm, which expresses a set of independent variables as a linear combination of factors, is a method of relating these factors to the properties of the samples for which the independent variables were obtained.

PSEUDO-ABSORBANCE, A, is represented by the equation:

$$A = -\log R = \log(1/R)$$

where R is the diffuse reflectance of the sample.

REFERENCE SPECTRUM—See *Background Spectrum*.

REFLECTANCE is described by the equation:

$$R = I/I_R$$

in which I is the intensity of radiation reflected from the surface of the sample and I_R is the intensity of radiation reflected from a background reference material and its incorporated losses due to solvent absorption, refraction, and scattering.

ROOT-MEAN-SQUARE (RMS) NOISE is calculated by the equation:

$$\mathrm{RMS} = \sqrt{\frac{1}{N} \times \sum_{i}^{N} \left(A_i - \overline{A}\right)^2}$$

in which A_i is the absorbance for each data point; $\overline{A}$ is the mean absorbance over the spectral segment; and N is the number of points per segment.

SPECTRAL REFERENCE LIBRARY is a collection of spectra of known materials for comparison with unknown materials. The term is commonly used in connection with qualitative methods of spectral analysis (e.g., identification of materials).

SPECULAR (SURFACE) REFLECTANCE is the reflectance of the front surface of the sample.

STANDARD ERROR OF CALIBRATION (SEC) is a measure of the capability of a model to fit reference data. SEC is the standard deviation of the residuals obtained from comparing the known values for each of the calibration samples to the values that are calculated from the calibration. SEC should not be used as an assessment tool for the expected method accuracy (trueness and

precision of prediction) of the predicted value of future samples. The method accuracy should generally be verified by calculating the standard error of prediction (SEP), using an independent validation set of samples. An accepted method is to mark a part of the calibration set as the validation set. This set is not fully independent but can be used as an alternative for the determination of the accuracy.

STANDARD ERROR OF CROSS-VALIDATION (SECV) is the standard deviation calculated using the leave-one-out method. In this method, one calibration sample is omitted from the calibration, and the difference is found between the value for this sample calculated from its reference value and the value obtained from the calibration calculated from all the other samples in the set. This process is repeated for all samples in the set, and the SECV is the standard deviation of the differences calculated for all the calibration samples. This procedure can also be performed with a group of samples. Instead of leaving the sample out, a group of samples is left out. The SECV is a measure of the model accuracy that one can expect when measuring future samples if not enough samples are available for the SEP to be calculated from a completely independent validation set.

STANDARD ERROR OF THE LABORATORY (SEL) is a calculation based on repeated readings of one or more samples to estimate the precision and/or accuracy of the reference laboratory method, depending on how the data were collected.

STANDARD ERROR OF PREDICTION (SEP) is a measure of model accuracy of an analytical method based on applying a given calibration model to the spectral data from a set of samples different from but similar to those used to calculate the calibration model. SEP is the standard deviation of the residuals obtained from comparing the values from the reference laboratory to those from the method under test for the specified samples. SEP provides a measure of the model accuracy expected when one measures future samples.

SURFACE REFLECTANCE, also known as *specular reflection,* is that portion of the radiation not interacting with the sample but simply reflecting back from the sample surface layer (sample–air interface).

TRANSFLECTION is a transmittance measurement technique in which the radiation traverses the sample twice. The second time occurs after the radiation is reflected from a surface behind the sample.

TRANSMITTANCE is represented by the equation:

$$T = I/I_0 \text{ or } T = 10^{-A}$$

in which I is the intensity of the radiation transmitted through the sample; I_0 is the intensity of the radiant energy incident on the sample and includes losses due to solvent absorption, refraction, and scattering; and A is the absorbance.

⟨1120⟩ RAMAN SPECTROSCOPY

INTRODUCTION

Raman is a vibrational spectroscopic technique and is therefore related to infrared (IR) and near-infrared (NIR) spectroscopy. The Raman effect itself arises as a result of a change in the polarizability of molecular bonds during a given vibrational mode and is measured as inelastically scattered radiation.

A Raman spectrum is generated by exciting the sample of interest to a virtual state with a monochromatic source, typically a laser. Light elastically scattered (no change in wavelength) is known as Rayleigh scatter and is not of interest in Raman spectrometry, except for marking the laser wavelength. However, if the sample relaxes to a vibrational energy level that differs from the initial state, the scattered radiation is shifted in energy. This shift is commensurate with the energy difference between the initial and final vibrational states. This "inelastically scattered" light is referred to as Raman scatter. Only about one in 10^6–10^8 photons incident on the sample undergoes Raman scattering. Thus lasers are employed in Raman spectrometers. If the Raman-scattered photon is of lower energy, it is referred to as Stokes scattering. If it is of higher energy, it is referred to as anti-Stokes scattering. In practice, nearly all analytically useful Raman measurements make use of Stokes-shifted Raman scatter.

The appearance of a Raman spectrum is much like an infrared spectrum plotted linearly in absorbance. The intensities, or the number of Raman photons counted, are plotted against the shifted energies. The x-axis is generally labeled "Raman Shift/cm^{-1}" or "Wavenumber/cm^{-1}". The Raman shift is usually expressed in wavenumber and represents the difference in the absolute wavenumber of the peak and the laser wavenumber. The spectrum is interpreted in the same manner as the corresponding mid-infrared spectrum. The positions of the (Raman shifted) wavenumbers for a given vibrational mode are identical to the wavenumbers of the corresponding bands in an IR absorption spectrum. However, the stronger peaks in a Raman spectrum are often weak in an IR spectrum, and vice versa. Thus the two spectroscopic techniques are often said to be complementary.

Raman spectroscopy is advantageous because quick and accurate measurements can often be made without destroying the sample (solid, semisolid, liquid or, less frequently, gas) and with minimal or no sample preparation. The Raman spectrum contains information on fundamental vibrational modes of the sample that can yield both sample and process understanding. The signal is typically in the visible or NIR range, allowing efficient coupling to fiber optics. This also means that a signal can be obtained from any medium transparent to the laser light; examples are glass, plastics, or samples in aqueous media. In addition, because Raman spectra are ordinarily excited with visible or NIR radiation, standard glass/quartz optics may be used.

General Chapters

From an instrumental point of view, modern systems are easy to use, provide fast analysis times (seconds to several minutes), and are reliable. However, the danger of using high-powered lasers must be recognized, especially when their wavelengths are in the NIR and, therefore, not visible to the eye. Fiber-optic probes should be used with caution and with reference to appropriate government regulations regarding lasers and laser classes.

In addition to "normal" Raman spectroscopy, there are several more specialized Raman techniques. These include resonance Raman (RR), surface-enhanced Raman spectroscopy (SERS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), Raman gain or loss spectroscopy, and hyper-Raman spectroscopy. These techniques are not widely employed in pharmaceutical laboratories, and are not addressed in this general information chapter.

QUALITATIVE AND QUANTITATIVE RAMAN MEASUREMENTS

There are two general classes of measurements that are commonly performed by Raman spectrometry: qualitative and quantitative.

Qualitative Raman Measurements

Qualitative Raman measurements yield spectral information about the functional groups that are present in a sample. Because the Raman spectrum is specific for a given compound, qualitative Raman measurements can be used as a compendial ID test, as well as for structural elucidation.

Quantitative Raman Measurements

For instruments equipped with a detector that measures optical power (such as Fourier transform [FT]-Raman spectrometers), quantitative Raman measurements utilize the following relationship between signal, S_ν, at a given wavenumber, ν, and the concentration of an analyte, C:

$$S_\nu = K\sigma_\nu(\nu_L - \nu\beta)^4 P_0 C$$

in which K is a constant that depends on laser beam diameter, collection optics, sample volume, and temperature; σ_ν is the Raman cross section of the particular vibrational mode; ν_L is the laser wavenumber; ν_β is the wavenumber of the vibrational mode; and P_0 is the laser power. The Raman cross section, σ_ν, is characteristic of the nature of the particular vibrational mode. The sample volume is defined by size of the focus of the laser beam at the sample, the optic being used for focusing, and the optical properties of the sample itself. Spot sizes at the sample can range from less than 1 µm for a microprobe to 6 mm for a large area sample system. For Raman spectrometers that measure the number of photons per second (such as change-coupled device [CCD]-Raman spectrometers) the corresponding equation is:

$$S_\nu = K\sigma_\nu \nu_L(\nu_L - \nu\beta)^3 P_0 C$$

From the above equations, it is apparent that peak signal is directly proportional to concentration. It is this relationship that is the basis for the majority of quantitative Raman applications.

FACTORS AFFECTING QUANTIFICATION

Sample-Based Factors

The most important sample-based factors that deleteriously affect quantitative Raman spectrometry are fluorescence, sample heating, absorption by the matrix or the sample itself, and the effect of polarization. If the sample matrix includes fluorescent compounds, the measured signal will usually contain a contribution from fluorescence. Fluorescence will be observed only if the laser excitation wavelength overlaps with an absorption band of a fluorescent compound. Fluorescence is typically observed as a broad sloping background underlying the Raman spectrum. Fluorescence can cause both a baseline offset and reduced signal-to-noise ratio. The wavelength range and intensity of the fluorescence is dependent on the chemical composition of the fluorescent material. Because fluorescence is generally a much more efficient process than Raman scattering, even very minor amounts of fluorescent impurities can lead to significant degradation of the Raman signal. Fluorescence can be reduced by using longer wavelength excitation sources such as 785 nm or 1064 nm. However, it should be remembered that the strength of the Raman signal is proportional to $(\nu_L - \nu_\beta)^4$, so the advantage of using a long-wavelength excitation laser to minimize fluorescence is at least partially offset by the reduced strength of the Raman signal. The greatest signal-to-noise ratio will be obtained by balancing fluorescence rejection, signal strength, and detector response.

Fluorescence in solids can sometimes be mitigated by exposing the sample to the laser radiation for a period of time before measurement. This process is called photobleaching, and operates by degrading the highly absorbing species. Photobleaching is less effective in liquids, where the sample is mobile, or if the amount of fluorescent material is more than a trace.

Sample heating by the laser source can cause a variety of effects, such as physical form change (melting), polymorph conversion, or sample burning. The chance for sample heating is greatest when the spot size at the sample is the smallest, i.e.,

when a microprobe is being used. This is usually an issue for colored, highly absorbing species, or very small particles that have low heat transfer. The effects of sample heating are usually observable either as changes in the Raman spectrum over time or by visual inspection of the sample. Besides decreasing the laser flux, a variety of methods can be employed to diminish laser-induced heating, such as moving the sample or laser during the measurement or improving the heat transfer from the sample with thermal contact or liquid immersion.

Absorption of the Raman signal by the matrix or the sample itself can also occur. This problem is more prevalent with long-wavelength FT-Raman systems where the Raman signal can overlap with an NIR overtone absorption. This effect will be dependent on the optics of the system as well as on the sample presentation. Associated with this effect is variability from scattering in solids as a result of packing and particle-size differences. The magnitude of all of these effects, however, is typically less severe than in NIR because of the limited depth of penetration and the relatively narrower wavelength region sampled in Raman spectroscopy.

Finally, it should be recognized that laser radiation is polarized and the Raman spectra of crystalline materials and other oriented samples can differ significantly depending on the way that the sample is mounted. If the Raman spectrometer is capable of producing linearly polarized radiation at the sample then a polarization scrambler is recommended for routine sample analysis.

Sampling Factors

Raman spectroscopy is a zero-background technique, in that the signal at the detector is expected to be zero in the absence of a sample. This situation can be contrasted with absorption spectrometry, where the signal at the detector is at a maximum in the absence of a sample. Zero-background techniques are inherently sensitive because small changes in sample concentration lead to proportionate changes in the signal level. The instrument will also be sensitive to other sources of light that can cause sample-to-sample variations in the measured signal level. In addition, a large background signal caused by fluorescence will lead to an increased noise level (photon shot noise). Thus it may be very difficult to use the absolute Raman signal for direct determination of an analyte. Other potential sources of variation are changes in the sample opacity and heterogeneity, changes in the laser power at the sample, and changes in optical collection geometry or sample position. These effects can be minimized by sampling in a reproducible, representative manner. Careful design of the instrumentation can reduce these effects but they cannot be eliminated entirely.

Use of an internal reference standard is the most common and robust method of eliminating variations caused by absolute intensity fluctuations. There are several choices for this approach. An internal standard can be deliberately added, and isolated peaks from this standard can be employed; or a band due to a moiety such as an aromatic ring, the Raman cross-section of which does not change with the way the sample is prepared, can also be used. For solution spectra, an isolated solvent band can be employed because the solvent will remain relatively unchanged from sample to sample. Also, in a formulation, an excipient peak can be used if it is in substantial excess compared to the analyte. The entire spectrum can also be used as a reference, with the assumption that laser and sample-orientation changes will affect the entire spectrum equally.

A second important sampling-based factor to consider is spectral contamination. Raman scattering is a weak effect that can be masked by a number of external sources. Common contamination sources include sample-holder artifacts (container or substrate) and ambient light. Typically, these issues can be identified and resolved by careful experimentation.

APPARATUS

Components

All modern Raman measurements involve irradiating a sample with a laser, collecting the scattered radiation, rejecting the Rayleigh-scattered light, differentiating the Raman photons by wavelength, and detecting the resulting Raman spectrum. All commercial Raman instruments therefore share the following common features to perform these functions:

1. Excitation source (laser)
2. Sampling device
3. Device to filter/reject light scattered at the laser wavelength
4. Wavelength processing unit
5. Detector and electronics

EXCITATION SOURCE (LASER)

Table 1 identifies several common lasers used for pharmaceutical applications or Raman spectrometry. UV lasers have also been used for specialized applications but have various drawbacks that limit their utility for general analytical measurements. As more applications for UV lasers are described, it is likely that they may become more common for Raman spectrometry.

General Chapters

Table 1. Lasers Used in Pharmaceutical Applications

Laser λ, nm (nearest whole number)	Type	Typical Power at Laser	Wavelength Range, nm (Stokes Region, 100 cm^{-1} to 3000 cm^{-1} shift)	Comments
NIR Lasers				
1064	Solid state (Nd:YAG)	Up to 3 W	1075–1563	Commonly used in Fourier transform instruments
830	Diode	Up to 300 mW	827–980	Typically limited to 2000 cm^{-1}; Raman shift because of CCD spectral response; less common than the other lasers
785	Diode	Up to 500 mW	791–1027	Most widely used dispersive Raman laser
Visible Lasers				
632.8	He–Ne	Up to 500 mW	637–781	Relatively small fluorescence risk
532	Doubled (Nd:YAG)	Up to 1 W	535–632.8	High fluorescence risk
514.5	Ar+	Up to 1 W	517–608	High fluorescence risk
488–632.8	Ar+	Up to 1 W	490–572	High fluorescence risk

SAMPLING DEVICE

Several sampling arrangements are possible, including direct optical interfaces, microscopes, fiber optic-based probes (either noncontact or immersion optics), and sample chambers (including specialty sample holders and automated sample changers). The sampling optics can also be designed to obtain the polarization-dependent Raman spectrum, which often contains additional information. Selection of the sampling device will often be dictated by the analyte and sample. However, considerations such as sampling volume, speed of the measurement, laser safety, and reproducibility of sample presentation should be evaluated to optimize the sampling device for any given application.

FILTERING DEVICE

The intensity of scattered light at the laser wavelength (Rayleigh) is many orders of magnitude greater than the Raman signal and must be rejected prior to the detector. Notch filters are almost universally used for this purpose and provide excellent rejection and stability combined with small size. The traditional use of multistage monochromators for this purpose, although still viable, is now rare. In addition, various filters or physical barriers to shield the sample from external radiation sources (e.g., room lights, laser plasma lines) may be required depending on the collection geometry of the instrument.

WAVELENGTH PROCESSING UNIT

The wavelength scale may be encoded by either a scanning monochromator, a grating polychromator (in CCD-Raman spectrometers) or a two-beam interferometer (in FT-Raman spectrometers). A discussion of the specific benefits and drawbacks of each of the dispersive designs compared to the FT instrument is beyond the scope of this chapter. Any properly qualified instruments should be suitable for qualitative measurements. However, care must be taken when selecting an instrument for quantitative measurements, as dispersion and response linearity might not be uniform across the full spectral range.

DETECTOR

The silicon-based CCD array is the most common detector for dispersive instruments. The cooled array detector allows measurements over the spectral range from 4500 to 100 cm^{-1} Raman shift with low noise when most visible lasers, such as frequency-doubled neodymium-doped yttrium–aluminum–garnet (Nd:YAG) (532 nm) or helium–neon (632.8 nm) lasers, are used. When a 785-nm diode laser is used, the wavelength range is reduced to about 3100 to 100 cm^{-1}. The most commonly used CCD has its peak wavelength responsivity when matched to the commonly used 632.8-nm He–Ne gas laser or 785-nm diode laser. FT instruments typically use single-channel germanium or indium–gallium–arsenide (InGaAs) detectors responsive in the NIR to match the 1064-nm excitation of a Nd:YAG laser.

Calibration

Raman instrument calibration involves three components: primary wavelength (x-axis), laser wavelength, and intensity (y-axis).

PRIMARY WAVELENGTH (X-AXIS)

In the case of FT-Raman instruments, primary wavelength-axis calibration is maintained, at least to a first approximation, with an internal He–Ne laser. Most dispersive instruments utilize atomic emission lamps for primary wavelength-axis calibration. In all instruments suitable for analytical Raman measurements, the vendor will offer a procedure of x-axis calibration that can be performed by the user. For dispersive Raman instruments, a calibration based on multiple atomic emission lines is preferred. The validity of this calibration approach can be verified subsequent to laser wavelength calibration by using a suitable Raman shift standard. For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.[1]

LASER WAVELENGTH

Laser wavelength variation can impact both the wavelength precision and the photometric (signal) precision of a given instrument. Even the most stable current lasers can vary slightly in their measured wavelength output. The laser wavelength must therefore be confirmed to ensure that the Raman shift positions are accurate for both FT-Raman or dispersive Raman instruments. A reference Raman shift standard material such as those outlined in ASTM E1840-96 (2002)[1] or other suitably verified materials can be utilized for this purpose. [NOTE—Reliable Raman shift standard values for frequently used liquid and solid reagents, required for wavenumber calibration of Raman spectrometers, are provided in the ASTM Standard Guide cited. These values can be used in addition to the highly accurate and precise low-pressure arc lamp emission lines that are also available for use in Raman instrument calibration.] Spectrometric grade material can be purchased from appropriate suppliers for this use. Certain instruments may use an internal Raman standard separate from the primary optical path. External calibration devices exactly reproduce the optical path taken by the scattered radiation. [NOTE—When chemical standards are used, care must be taken to avoid contamination and to confirm standard stability.]

Unless the instrument is of a continuous calibration type, the primary wavelength axis calibration should be performed, as per vendor procedures, just prior to measuring the laser wavelength. For external calibration, the Raman shift standard should be placed at the sample location and measured using appropriate acquisition parameters. The peak center of a strong, well-resolved band in the spectral region of interest should be evaluated. The position can be assessed manually or with a suitable, valid peak-picking algorithm. The software provided by the vendor might measure the laser wavelength and adjust the laser wavelength appropriately so that this peak is at the proper position. If the vendor does not provide this functionality, the laser wavelength should be adjusted manually. Depending on the type of laser, the laser wavelength can vary with temperature, current, and voltage. Wavelength tolerances can vary depending on the specific application.

SIGNAL LEVEL (Y-AXIS)

Calibration of the photometric axis can be critical for successful quantification by using certain analytical methods (chemometrics) and method transfer between instruments. Both FT-Raman and dispersive Raman spectrometers should undergo similar calibration procedures. The tolerance of photometric precision acceptable for a given measurement should be assessed during the method development stage.

To calibrate the photometric response of a Raman instrument, a broad-band emission source should be used. There are two accepted methods. *Method A* utilizes a tungsten white light source.[2] The output power of such sources is traceable to the National Metrology Institute (NMI). In the United Kingdom, the National Physical Laboratory also provides calibrated light bulbs. Several other vendors also provide NIST-traceable irradiance calibration standards. This method is applicable to all common laser excitation wavelengths listed in *Table 1*. In *Method B*, NIST standard reference materials (SRMs) are utilized.[3] Several doped-glass fluorescence standards are currently available.

Method A—The source should be placed at the sample location with the laser off and the response of the detector measured (using parameters appropriate for the instrument). The output for the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach.

Method B—The fluorescence standard should be placed at the sample location. With the laser on, a spectrum of the SRM should be obtained (using parameters appropriate for the instrument). The output of the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This

[1] ASTM E1840-96 (2002) *Standard Guide for Raman Shift Standards for Spectrometer Calibration*, ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA, USA 19428-2959.

[2] NIST-traceable tungsten white light source statement: While the calibration of the Raman frequency (or Raman shift, cm^{-1}) axis using pure materials and an existing ASTM standard is well accepted, techniques for calibration of the Raman intensity axis are not. Intensity calibrations of Raman spectra can be accomplished with certified white light sources.

[3] NIST SRM 2241: Ray KG, McCreery RL. Raman intensity correction standard for systems operating with 785-nm excitation. *Appl. Spectrosc.* 1997, 51, 108–116.

correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach. [NOTE—*Method B* is currently appropriate for systems with 785-nm (SRM 2241), 532-nm (SRM 2242), and both 514.5-nm and 488-nm (SRM 2243) laser excitation. NIST is currently developing other SRMs that will be wavelength-specific for 1064-nm (SRM 2244) and 632.8-nm excitation (expected to be available in 2006).]

EXTERNAL CALIBRATION

Detailed functional validation employing external reference standards is recommended to demonstrate instrumental suitability for laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality control procedures; rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis or purpose. For instruments installed in a process location or in a reactor where positioning of an external standard routinely is not possible, including those instruments that employ an internal calibration approach, the relative performance of an internal versus an external calibration approach should be periodically checked. The purpose of this test is to check for changes in components that might not be included in the internal calibration method (process lens, fiber-optic probe, etc.), e.g., photometric calibration of the optical system.

QUALIFICATION AND VERIFICATION OF RAMAN SPECTROMETERS

The suitability of a specific instrument for a given method is ensured by a thorough technology-suitability evaluation for the application; a routine, periodic instrument operational qualification; and the more frequent performance verification (see *Definition of Terms and Symbols*). The purpose of the technology-suitability evaluation is to ensure that the technology proposed is suitable for the intended application. The purpose of the instrument qualification is to ensure that the instrument to be used is suitable for its intended application and, when requalified periodically, continues to function properly over extended time periods. When the device is used for a specific qualitative or quantitative analysis, regular performance verifications are made. Because there are many different approaches to measuring Raman spectra, instrument operational qualification and performance verification often employ external standards that can be used on any instrument. As with any spectrometric device, a Raman instrument needs to be qualified for both wavenumber (x-axis and shift from the excitation source) and photometric (signal axis) precision.

In performance verification, a quality-of-fit to an initial scan or group of scans (often referred to in nonscanning instruments as an accumulation) included in the instrumental qualification can be employed. In such an analysis, it is assumed that reference standard spectra collected on a new or a newly repaired, properly operating instrument represent the best available spectra. Comparison of spectra taken over time on identical reference standards (either the original standard or identical new standards, if stability of the reference standards is a concern) forms the basis for evaluating the long-term stability of a Raman measurement system.

Frequency of Testing

Instrumental qualification is performed at designated intervals or following a repair or significant optical reconfiguration, such as the replacement of the laser, the detector or the notch or edge filters. Full instrument requalification might not be necessary when changing between sampling accessories such as a microprobe, a sample compartment, or a fixed fiber-optic probe. Performance verification tests may be sufficient in these cases; instrument-specific guidance from the vendor on qualification requirements should be followed. Tests include wavelength (x-axis and shift from the excitation source) and photometric (signal axis) precision. Instrument qualification tests require that specific application-dependent tolerances be met.

Performance verification is carried out on the instrument configured for the analytical measurements and is performed more frequently than instrument qualification. Performance verification includes measurement of the wavelength uncertainty and intensity-scale precision. Wavelength precision and intensity-scale precision tests may be needed prior to any data collection on a given day. Performance is verified by matching the current spectra to those collected during the previous instrument qualification.

Instrument Operational Qualification

It is important to note that the acceptance specifications given in both the *Instrument Operational Qualification* and *Performance Qualification* sections are applicable for general use; specifications for particular instruments and applications can vary depending on the analysis method used and the desired accuracy of the final result. ASTM standard reference materials are also specified, with the understanding that under some circumstances (specifically remote on-line applications) calibration using one of these materials may be impractical, and other suitably verified materials can be employed. At this juncture it is important to note that specific parameters such as spectrometer noise, limits of detection (LOD), limits of quantification (LOQ), and

acceptable spectral bandwidth for any given application should be included as part of the analytical method development. Specific values for tests such as spectrometer noise and bandwidth will be dependent on the instrument chosen and the purpose required. As a result, specific instrument tests for these parameters are not dictated in this information chapter.

WAVELENGTH (X-AXIS) ACCURACY

It is important to ensure the accuracy of the wavelength axis via calibration to maintain the integrity of Raman peak positions. Wavelength calibration of a Raman spectrometer consists of two parts: primary wavelength axis and laser wavelength calibration. After both the primary wavelength axis and the laser wavelength are calibrated, instrument wavelength uncertainty can be determined. This can be accomplished using a Raman shift standard such as the ASTM shift standards or other suitably verified material. Selection of a standard with bands present across the full Raman spectral range is recommended so that instrument wavelength uncertainty can be evaluated at multiple locations within the spectrum. The tolerance of wavelength precision that is required for a given measurement should be assessed during the method-development stage. [Note—For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.]

PHOTOMETRIC PRECISION

Laser variation in terms of the total emitted photons occurring between two measurements can give rise to changes in the photometric precision of the instrument. Unfortunately, it is very difficult to separate changes in the photometric response associated with variations in the total emitted laser photons from the sample- and sampling-induced perturbations. This is one of the reasons why absolute Raman measurements are strongly discouraged and why the photometric precision specification is set relatively loosely. The tolerance of photometric precision required for a given measurement should be assessed during the method-development stage.

PERFORMANCE QUALIFICATION

The objective of performance qualification is to ensure that the instrument is performing within specified limits with respect to wavelength precision, photometric axis precision, and sensitivity. In certain cases when the instrument has been set up for a specific measurement (for example, installed in a process reactor), it might no longer be possible or desirable to measure the wavelength and photometric (signal) qualification reference standards identified above. Provided instrument operational qualification has shown that the equipment is fit for use, a single external performance verification standard can be used to reverify function on a continuing basis (for example, a routinely used process solvent signal, for both wavelength and photometric precision, following reactor cleaning). The performance verification standard should match the format of the samples in the current analysis as closely as possible and use similar spectral acquisition parameters. Quantitative measurements of an external performance verification standard spectrum check both the wavelength (x-axis and laser wavelength) and the photometric (signal) precision. Favorable comparison of a series of performance verification spectra demonstrates proper continued operation of the instrument.

WAVELENGTH PRECISION

The wavelength precision should be measured by collecting data for a single spectrum of the selected Raman shift standard for a period equal to that used in the photometric consistency test. When appropriate, powdered samples should be repacked between each set of measurements. Peak positions across the spectral range of interest are used to calculate precision. Performance is verified by matching the current peak positions to those collected during the previous instrument qualification and should not vary with a standard deviation of more than $\pm 0.3\ cm^{-1}$, although this specification can be adjusted according to the required accuracy of the measurement.

PHOTOMETRIC PRECISION

The photometric precision should be measured by collecting data for a single spectrum of a suitably verified reference standard material for a specified time. After suitable baseline correction, the areas of a number of bands across the spectral range of interest should be calculated by means of an appropriate algorithm. The area of the strongest band is set to 1, and all other envelopes are normalized to this band. Performance is verified by matching the current band areas to the respective areas collected during the previous instrument qualification. The areas should vary by no more than 10%, although this specification can be adjusted according to the required accuracy of the measurement.

LASER POWER OUTPUT PRECISION AND ACCURACY

This test is applicable only to Raman instruments with automatic, internal laser power meters. Instruments without laser power measurement should utilize a calibrated laser power meter from a reputable supplier. The laser output should be set to a representative output, dictated by the requirements of the analytical measurement and the laser power measured. The output should be measured and checked against the output measured at instrument qualification. The power (in milliwatts or watts) should vary by no more than 25% compared to the qualified level. If the power varies by more than this amount, the instrument should be serviced (as this variation might indicate, among other things, a gross misalignment of the system or the onset of failure of the laser).

For instruments with an automatic, internal laser power meter, the accuracy of the values generated from the internal power meter should be compared to a calibrated external laser power meter at an interval of not more than 12 months. The internally calculated value should be compared to that generated by the external power meter. Performance is verified by matching the current value to that generated during the previous instrument qualification. The manufacturer might provide software to facilitate this analysis. If the instrument design prevents the use of an external power meter, then the supplier should produce documentation to ensure the quality of the instrument and provide a recommended procedure for the above analysis to be accomplished during a scheduled service visit.

METHOD VALIDATION

Validation of Raman methods will follow the same protocols described in *Validation of Compendial Procedures* ⟨1225⟩ in terms of accuracy, precision, etc. However, several of these criteria are affected by variables specific to Raman spectrometry. Fluorescence is the primary variable that can affect the suitability of a method. The presence of fluorescent impurities in samples can be quite variable and have little effect on the acceptability of a material. The method must be flexible enough to accommodate different sampling regimes that may be necessary to minimize the effects of these impurities.

Detector linearity must be confirmed over the range of possible signal levels. Fluorescence might drive both the signal baseline and the noise higher than that used in the validation, in which case the fluorescence must be decreased, or the method modified to accommodate the higher fluorescence levels. This is also true for the precision, limit of detection, and limit of quantification of the method, as increased baseline noise will negatively impact all of these values. Because fluorescence can also affect quantification caused by baseline shifts, acceptable quantification at different levels of photobleaching, when used, should also be confirmed.

The impact of the laser on the sample must be determined. Visual inspection of the sample and qualitative inspection of the Raman spectrum for measurements with differing laser powers and exposure times will confirm that the sample is not being altered (other than by photobleaching). Specific variables to confirm in the spectrum are shifts in peak position, changes in peak height and band width, and unexpected changes in background intensity.

Method precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids, and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample holder geometry, but will vary from instrument to instrument based on excitation and collection optical configuration.

DEFINITION OF TERMS AND SYMBOLS

CALIBRATION MODEL is a mathematical expression that relates the response from an analytical instrument to the properties of samples.

INSTRUMENT BANDPASS (OR RESOLUTION) is a measure of the capability of a spectrometer to separate radiation of similar wavelengths.

OPERATIONAL QUALIFICATION is the process by which it is demonstrated and documented that the instrument performs according to specifications, and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, major repair, etc.

PERFORMANCE QUALIFICATION is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Qualification may employ the same or different standards for different performance characteristics.

RAMAN SPECTRA[4] are plots of the radiant energy, or number of photons, scattered by the sample through the indirect interaction between the molecular vibrations in the sample and monochromatic radiation of frequency much higher than that of the vibrations. The abscissa is usually the difference in wavenumber between the incident and scattered radiation.

(NORMAL) RAMAN SCATTERING[4] is the inelastic scattering of radiation that occurs because of changes in the polarizability, of the relevant bonds during a molecular vibration. Normal Raman spectra are excited by radiation that is not in resonance with electronic transitions in the sample.

[4] Chalmers, J., Griffiths, P., Eds. *Handbook of Vibrational Spectroscopy;* John Wiley & Sons, Ltd: New York, 2002.

General Chapters

RAMAN WAVENUMBER SHIFT[4],

$$\Delta\tilde{\nu}$$

is the wavenumber of the exciting line minus the wavenumber of the scattered radiation. SI unit: m^{-1}. Common unit: cm^{-1} = 100 m^{-1}.

$$\beta\Delta\tilde{\nu}$$

where β is the differential Raman cross section, is positive for Stokes scattering and negative for anti-Stokes scattering.

⟨1121⟩ NOMENCLATURE

INTRODUCTION

The *USP* (or *NF*) titles for monograph articles are legally recognized under the Federal Food, Drug, and Cosmetic Act as the designations for use in labeling the articles to which they apply.

The value of designating each drug substance by one and only one nonproprietary[1] name is important in terms of achieving simplicity and uniformity in drug nomenclature. In support of the U.S. Adopted Names program (see *Mission and Preface* in *USP–NF*), of which the U.S. Pharmacopeial Convention is a co-sponsor, the USP Council of Experts gives consideration to the adoption of the U.S. Adopted Name, if any, as the official title for any compound that attains compendial recognition.

A compilation of the U.S. Adopted Names (USAN) published from the start of the USAN program in 1961, as well as other names for drugs, both current and retrospective, is provided in the *USP Dictionary of USAN and International Drug Names.* This publication serves as a book of names useful for identifying and distinguishing all kinds of names for drug substances, whether public, proprietary, chemical, or code-designated names.[2]

A nonproprietary name of a drug serves numerous and varied purposes. Its principal function is to identify the substance to which it applies by means of a designation that may be used by the professional and lay public free from the restrictions associated with registered trademarks. Teaching in the health sciences requires a common designation, especially for a drug that is available from several sources or is incorporated into a combination drug product; nonproprietary names facilitate communication among healthcare providers; nonproprietary names must be used as the titles of the articles recognized by official drug compendia; a nonproprietary name is essential to the pharmaceutical manufacturer as a means of protecting trademark rights in the brand name for the article concerned; and, finally, the manufacturer is obligated by federal law to include the established nonproprietary name in advertising and labeling.

Under the terms of the Drug Amendments of 1962 to the Federal Food, Drug, and Cosmetic Act, which became law October 10, 1962, the Secretary of Health and Human Services is authorized to designate an official name for any drug wherever deemed "necessary or desirable in the interest of usefulness and simplicity."[3]

The Commissioner of Food and Drugs and the Secretary of Health and Human Services published in the *Federal Register* regulations effective November 26, 1984, which state, in part:

> **"Sec. 299.4 Established names of drugs."**
>
> "(e) The Food and Drug Administration will not routinely designate official names under section 508 of the act. As a result, the established name under section 502(e) of the act will ordinarily be either the compendial name of the drug or, if there is no compendial name, the common and usual name of the drug. Interested persons, in the absence of the designation by the Food and Drug Administration of an official name, may rely on as the established name for any drug the current compendial name or the USAN adopted name listed in *USAN and the USP Dictionary of Drug Names.*"[4]

It will be noted that the monographs on the biologics, which are produced under licenses issued by the Secretary of the U.S. Department of Health and Human Services, represent a special case. Although efforts continue toward achieving uniformity, there may be a difference between the respective title required by federal law and the *USP* title. Such differences are fewer than in past revisions of the Pharmacopeia. The *USP* title, where different from the FDA Center for Biologics Evaluation and Research title, does not necessarily constitute a synonym for labeling purposes; the conditions of licensing the biologic concerned require that each such article be designated by the name appearing in the product license issued to the manufacturer. Where a *USP* title differs from the title in the federal regulations, the former has been adopted with a view to usefulness, simplicity, and conformity with the principles governing the selection of monograph titles generally.

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[1] The term "generic" has been widely used in place of the more accurate and descriptive term "nonproprietary" with reference to drug nomenclature.

[2] *USP Dictionary of USAN and International Drug Names* is obtainable on order from U.S. Pharmacopeia, Customer Service Department, 12601 Twinbrook Parkway, Rockville, MD 20852.

[3] F.D.&C. Act, Sec. 508 [358].

[4] 53 Fed. Reg. 5369 (1988) amending 21 CFR § 299.4.

MONOGRAPH NAMING POLICY FOR SALT DRUG SUBSTANCES IN DRUG PRODUCTS AND COMPOUNDED PREPARATIONS

The titles of *USP* monographs for drug products and compounded preparations formulated with a salt of an acid or base use the name of the active moiety, as defined below. The strength of the product or preparation is also expressed in terms of the active moiety.

An active moiety is the molecule or ion, excluding those appended portions of the molecule that cause the drug to be a salt (including a salt with hydrogen or coordination bonds), or other noncovalent derivative (such as a complex, chelate, or clathrate) of the molecule. The active moiety is responsible for the physiological or pharmacological action of the drug substance, without regard to the actual charged state of the molecule in vivo. For example, the active moiety of a hydrochloride salt of a base is the free base and not the protonated form of the base. The active moiety of a metal salt of an acid is the free acid.

This Policy is followed by USP in naming drug products and compounded preparations that are newly recognized in the *USP*. Revising existing monographs to conform to this Policy is not intended, except where the USP Council of Experts determines that, for reasons such as safety, a nomenclature change is warranted.

Labeling

The labeling clearly states the specific salt form of the active moiety that is present in the product or preparation because this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (when applicable) are provided in the labeling.

Exceptions

In rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this policy may be considered. In such cases, when the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation also is expressed in terms of the specific salt form.

GENERAL NOMENCLATURE FORMS

List of the specific drug products with extensive examples can be found in the USP Nomenclature Guidelines (www.usp.org). USP also developed some general practices for drug product nomenclature:

- The [ROUTE OF ADMINISTRATION] is omitted for those dosage forms for which the route of administration is understood. The general form then becomes simply [DRUG] [DOSAGE FORM].
  - — Thus, oral will not be included as the route of administration for orally administered capsules, tablets, and lozenges.
  - — The route of administration is omitted for topically applied products—creams, ointments, lotions, and pastes. However, if some other route of administration is intended (e.g. ophthalmic), it will be included in the monograph title.
- In some instances, the drug is supplied in one dosage form for the preparation of the intended dosage form. In such cases, the dosage form provided in the container is named first and the word "for" appears, followed by the final dosage form that is suitable for administration. The general format becomes [DRUG] [DOSAGE FORM] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM]., e.g. Aspirin Effervescent Tablets for Oral Solution.
- The term "for" is included in the names of solid preparations which must be dissolved or suspended in a suitable liquid to obtain a dosage form suitable for administration, and the general format becomes [DRUG] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM]. e.g. Ampicillin for Oral Suspension, Cytarabine for Injection.
- The term "Vaginal Inserts", rather than "Vaginal Tablets", "Vaginal Capsules", or "Vaginal Suppositories" is used in the title of this type of vaginal preparation to decrease the potential for misadministration of these products.
- The term "Suppositories" is used in the titles of solid preparations that are intended for rectal administration.
- Solutions administered by injection are officially titled Injections (see *Injections and Implanted Drug Products* 〈1〉). The route of administration is omitted for drugs that are injected, because the route (e.g. intravenous, intramuscular, subcutaneous, etc.) must appear on the labels and in the labeling. USP defines seven types of injections:
  1. [DRUG] Injection—Liquid preparations that are drug substances or solutions thereof.
  2. [DRUG] for Injection—Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for Injections.
  3. [DRUG] Injectable Emulsion—Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.
  4. [DRUG] Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium.
  5. [DRUG] for Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Injectable Suspensions.
  6. [DRUG] Extended-Release Injectable Suspension—Liquid preparations of solids suspended in a suitable liquid medium and formulated in a manner that allows the contained API to be available over an extended period of time.

7. [DRUG] for Extended-Release Injectable Suspension—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Extended-Release Injectable Suspensions.

Injections intended to be administered through an intravenous secondary line ("piggyback") formulated in vehicles other than Water for Injection shall be named [DRUG] in [VEHICLE]. Examples of Vehicle formats which currently appear in *USP* monograph titles are:

1. [DRUG] in Dextrose Injection
2. [DRUG] in Dextrose and Sodium Chloride Injection
3. [DRUG] in Lactated Ringer's and Dextrose Injection
4. [DRUG] in Sodium Chloride Injection

POLICY FOR IMPLEMENTATION OF NOMENCLATURE REVISIONS

It is the practice of USP to set the official dates of nomenclature revisions (change of the established name or nomenclature aspects of the labeling sections in the monographs) to allow a reasonable time for product label changes to be made and to allow health practitioners and consumers time to become familiar with the new terminology. The assignment of an implementation schedule is handled by the USP Expert Committee. USP's implementation schedule, shown below, is automatic, unless an exception is sought.

18 Months

Implementation period of 18 months is usually applied when only one or a small number of products is affected.

30 Months

Implementation period of 30 months is usually applied when names or labeling of multisource products or multiproduct lines of a firm's preparations are being changed.

60 Months

Implementation period of 60 months is usually applied for title and labeling changes that affect excipients, because such changes would require relabeling of very large numbers of prescription-only and OTC preparations.

There may be exceptions to this schedule where a shorter time is needed in order to specify nomenclature and labeling changes in cases where public health and safety are a concern. Extensions to the implementation schedule are rarely made, and must have suitable justification as well as the approval of the USP Expert Committee. Any questions or concerns regarding this postponement schedule may be addressed to the USP staff liaison assigned to the Expert Committee.

⟨1125⟩ NUCLEIC ACID-BASED TECHNIQUES—GENERAL

SCOPE

Nucleic acid-based assays are used in a variety of settings, the most common of which include the detection of infectious agents (viruses, bacteria, etc.), and cellular materials, as well as disease profiling. More recently such assays have also been used for forensic purposes and for the detection of trace contamination in biological materials. The latter include pharmaceutical development applications, such as viral clearance and adventitious agent testing in vaccine seed lots and tissue culture cell banks. This chapter introduces a series of general information chapters that provide techniques that support procedures for the detection and analysis of nucleic acids (see *Figure 1*). The assays using these techniques may be presented in a USP general chapter or in a private specification.

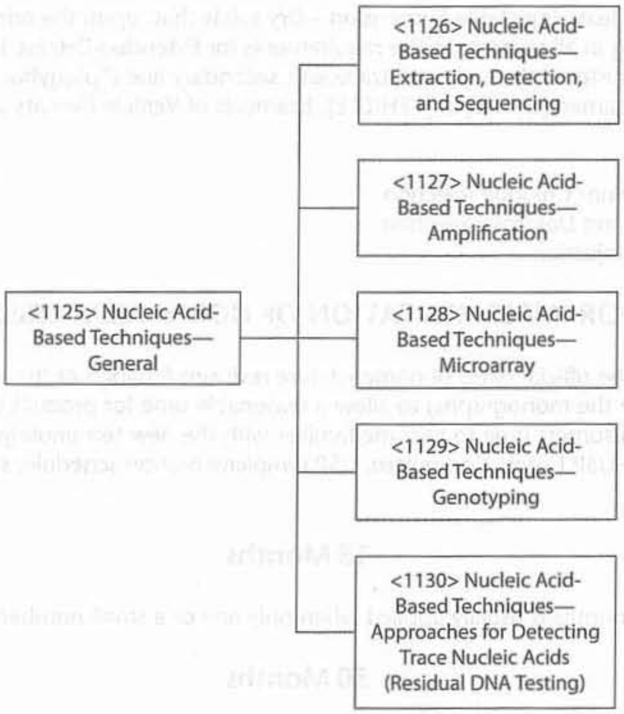


Figure 1

The major requirement for any nucleic acid analytical procedure is the availability of pure, intact nucleic acids for analysis. The information in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* 〈1126〉 discusses procedures available for nucleic acid extraction and handling. Hybridization is the core mechanism underlying many molecular biology techniques, and in addition to the detection of nucleic acids by absorbance and fluorescence measurements and size measurement by gel electrophoresis, this chapter also covers blotting and identification of nucleic acid species by hybridization assays using labeled probes. Hybridization probes are oligonucleotides that have a sequence that is complementary to the target of interest. Probes contain radioactive, fluorescent, biotin, digoxigenin, or other tags that, upon binding of the probe to the target, allow visualization and identification of the target. Probes are capable of detecting target sequences that are present in concentrations too low to be detected by absorbance measurements or gel electrophoresis.

These analytical procedures require a minimum quantity of nucleic acid, typically in the nanogram to microgram range. However, in the vast majority of cases, e.g., in the detection of viruses or rare cellular RNA species, the nucleic acid under assay is present in minute quantities (in the picogram to femtogram range), and an amplification step must be performed before the nucleic acid can be detected and identified. The amplification step may be directed either at the signal used for detection (signal amplification), such as the branched DNA assay (bDNA assay), or at the target as in nucleic acid amplification technologies (NAT).

In 1983 a revolutionary yet simple process termed polymerase chain reaction (PCR) was developed for amplifying the number of specific nucleic acid fragments present in a sample, and in just a few years after its discovery PCR became the most frequently used procedure for amplifying nucleic acids, especially DNA. Since the inception of PCR, the number of applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. Numerous variations of assay procedures have been developed for specific analytes. The general information chapter, *Nucleic Acid-Based Techniques—Amplification* 〈1127〉, describes amplification procedures used for DNA and RNA analysis as well as qualitative and quantitative NAT assays. Signal amplification procedures in which the signals, typically fluorescent signals, are used to detect the nucleic acid of interest, are not very common. The major signal amplification procedure, the branched DNA or bDNA assay, is used predominantly for viral nucleic acid detection.

Quality assurance aspects of the methodology are also covered, together with a summary of current regulatory requirements for NAT assays. The need for globally comparable, accurate, and reliable results in the diagnostics field has driven the quest for, and development of, national and international standards within an increasingly sophisticated and metrologically sound, highly developed international regulatory environment devoted to the highest standards of regulatory science. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT. The general information chapter, *Nucleic Acid-Based Techniques—Microarrays* 〈1128〉, addresses a still-emerging field that is of increasing relevance to molecular DNA analysis. Detailed treatment of various microarrays, including data analysis and validation, are excluded from 〈1128〉 at this time. The general information chapter, *Nucleic Acid-Based Techniques—Genotyping* 〈1129〉, focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences and common genetic variations, e.g., single nucleotide polymorphisms (SNPs). The final general information chapter in the

series, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* 〈1130〉, describes residual DNA testing in the context of pharmaceutical manufacturing. Applications relevant to viral adventitious agents, however, are discussed in the general information chapter *Virology Test Methods* 〈1237〉.

Two major uses of nucleic acid testing are excluded from this family of NAT chapters: viral testing for blood and blood product safety and genetic testing. The traditional perspective of USP is to develop public standards that can be applied to a particular final product without expressively defining a product and/or its production details. This chapter aims to specify when traditional methodologies or existing standards can be adapted. Novel methodologies for amplification and detection by NAT are also highlighted. As these new methodologies become mature and properly validated, they will be included in subsequent revisions.

Due to rapid development in the field, compendial and regulatory affairs scientists are advised to consult the current edition of *USP* and its *Supplements* regularly.

GLOSSARY

3′–5′ Exonuclease activity: Enzymatic activity to remove a mispaired nucleotide from the 3′ end of the growing strand. The reaction is a hydrolysis of a phosphoester bond. The presence of a 3′–5′ exonuclease, or proofreading, activity improves the fidelity of the polymerization.

5′–3′ Exonuclease activity: Enzymatic activity to remove a mispaired nucleotide from the 5′ end of a polynucleotide strand. This activity is actually that of a single-strand-dependent endonuclease and is needed to remove RNA primers of Okazaki fragments, the RNA strand in the intermediate DNA–RNA heteroduplex during reverse transcription, and during DNA repair.

Absorbance: [Symbol: A]The logarithm, to the base 10, of the reciprocal of the transmittance (T). [NOTE—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

Accuracy: The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value.

Allele: One of two or more alternative forms of a gene at a given position (locus) on a chromosome, caused by a difference in the sequence of DNA.

Amplicon: A short segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers. Sometimes referred to as an amplimer.

Amplification: The enzymatic in vitro replication of a target nucleic acid.

Annealing: Hybridizing or binding of complementary nucleic acids, usually at an optimal temperature.

Concatenation: The process in which a DNA segment composed of repeated sequences is linked end-to-end.

Complementary dna (cdna): DNA synthesized from an RNA template in an enzymatic reaction catalyzed by the enzyme reverse transcriptase.

Denaturation: The process of separating double-stranded DNA into single strands by breaking the hydrogen bonds. This is typically accomplished by heating the DNA solution to temperatures greater than 90° or by treating it with strong alkali.

Deoxyribonucleic acid (dna): The genetic material that is passed from parent to daughter cells and propagates the characteristics of the species in the form of genes it contains and the proteins for which it codes. DNA contains the following four deoxyribonucleosides: dA, dC, dT, and dG.

Deoxyribonucleotide triphosphate (dntp): A base that is added to a primer during the PCR that comprises the newly synthesized strand. Examples of dNTPs are dATP, dUTP, dCTP, dGTP, and dTTP.

Detection limit: It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

DNA polymerase: An enzyme that can synthesize new complementary DNA strands using a DNA template and primer. Several of these enzymes are commercially available, including *Taq* DNA polymerase and rTth DNA polymerase.

Endonuclease: An enzyme that cleaves phosphodiester bonds in a polynucleotide chain.

Energy transfer: This describes the process in which an excited state of one molecular entity (the donor) is deactivated to a lower-lying state by transferring energy to a second molecular entity (the acceptor), which is thereby raised to a higher energy state.

Extension: Refers to the elongation of the DNA chain that is being synthesized using the parent DNA strand as the template for synthesis of that daughter strand. This is a natural process that occurs during DNA replication. Extension occurs during the PCR process with DNA polymerases.

Extinction coefficient: [Symbol: ε]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles/L, of the substance and the absorption path length, in cm. [NOTE—Terms formerly used include molar absorbancy index; molar absorptivity; and molar absorption coefficient.]

Fidelity: Fidelity is a measure of the accuracy of nucleic acid replication. The polymerase enzyme used is only one of the elements that influences fidelity. Other elements include buffer conditions, thermal cycling parameters, number of cycles, efficiency of amplification, and the sequence of the DNA being copied.

Fluorophore: A functional group in a molecule that makes the molecule fluorescent by absorbing energy of a specific wavelength and re-emits the energy at another wavelength.

Fluorescence: The emission of one or more photons by a molecule or atom activated by the absorption of a quantum of electromagnetic radiation. X-rays, UV, visible light, and IR radiations may all stimulate fluorescence. For details on the spectroscopic measurement of fluorescence, see *Fluorescence Spectroscopy* 〈853〉.
Genome: The complete genetic complement or the complete set of instructions for reproducing an organism and carrying out its biological function in life. The DNA in our cells comprises our genome. When our cells divide, the complete genome in our cells is duplicated for transmission to each of the remaining daughter cells.
Genotype: The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e., the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.
Genotyping: The process of assessing genetic variations present in an individual.
Hairpin: Antiparallel duplex structure that forms by pairing of inverted repeat sequences within a single-stranded nucleic acid. The helical section is called the stem and the unpaired base segment at the end of the structure is called the loop.
Hot-start PCR: Technique that is commonly used to improve the sensitivity and specificity of PCR amplification. A hot start is performed by withholding from the reaction mix a key component necessary for amplification until the reaction reaches a temperature above the optimal annealing temperature of the primers. The component withheld from the reaction mix can be primers, DNA polymerase, $MgCl_2$, or dNTPs.
Hybridization: The process of forming a double-stranded nucleic acid molecule, for example between a nucleotide sequence (probe) and a target.
Ligation: The process of joining two or more DNA fragments.
Melting temperature (T_m): The temperature at which 50% of the DNA becomes single-stranded.
Microarray: Sets of miniaturized chemical reaction areas that are used to test DNA fragments, antibodies, or proteins. Usually the probes are immobilized on a chip and hybridized with target.
Mismatch: Unconventional base pairing (other than C with G, and A with T or U). A mismatched base pair has lower bonding energy and decreases the stability of the DNA molecule.
Nucleic acid: Linear polymers of nucleotides, linked by 3′, 5′ phosphodiester linkages. In DNA, deoxyribonucleic acid, the sugar group is deoxyribose, and the bases consist of adenine, guanine, thymine, and cytosine. RNA, ribonucleic acid, has ribose as the sugar, and uracil replaces thymine.
Oligonucleotide: Linear sequence comprising as many as 25 nucleotides joined by phosphodiester bonds, generally used as a DNA synthesis primer.
Photobleaching: Photobleaching is the irreversible destruction of a fluorophore in the excited state. Different fluorophores have different rates of photobleaching. For example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. A simple and practical way to overcome this is to reduce the incident radiation.
Polymerase: An enzyme that catalyzes the synthesis of nucleic acids on pre-existing nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.
Polymerase chain reaction (PCR): A laboratory technique that rapidly amplifies a specific region of double-stranded DNA, predetermined by the pair of primers used for amplification. Generally involves the use of a heat-stable DNA polymerase.
Precision: The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample.
Primer: Nucleic acid polymerases link a mononucleotide to a chain of nucleic acids, which is called the primer. RNA polymerases are able to use a single nucleotide as a primer, but DNA polymerases always require an oligonucleotide.
Probe: A specific DNA or RNA sequence that has been labeled by radioactive, fluorescent, or chemiluminescent tags and is used to detect complementary sequences by hybridization techniques such as blotting or colony hybridization. In addition, probes can also be used for quantitation of amplicons as described for quantitative PCR assays. A more detailed description of such probes is given in the general information chapter, *Nucleic Acid-Based Techniques—Amplification* 〈1127〉.
Processivity: The ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate.
Proofreading activity: Literally to read for the purpose of detecting errors for later correction. DNA polymerase has a 3′ to 5′ exonuclease activity that is used during polymerization to remove recently added nucleotides that are incorrectly paired.
Quantitation limit: It is the lowest amount of analyte in a sample that can be determined with an acceptable precision and accuracy under the stated experimental conditions.
Quenching: The process of extinguishing, removing, or diminishing a physical property such as heat or light. Fluorescence quenching can be either collisional or static.
Reverse transcriptase: An enzyme that requires a DNA primer and catalyzes the synthesis of a DNA strand from an RNA template. An enzyme that can use RNA as a template to synthesize DNA.
Reverse transcription (rt): The process of making cDNA using an RNA template.
Real-time PCR: May often be referred to as Quantitative PCR or Real-Time Quantitative PCR but not RT-PCR and is a procedure for simultaneous DNA quantitation and amplification. The generation of amplicons monitored as they are generated by the use of a fluorescent reporter system and captured by sophisticated instrumentation.
Real-time (RT-PCR): The combination of real-time PCR and reverse transcription PCR.
Reverse transcriptase polymerase chain reaction (RT-PCR): A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.
Ribonucleic acid (RNA): A type of nucleic acid composed of a specific sequence of ribonucleotides linked together. RNA contains the following four ribonucleosides: A, C, G, and U.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage.
rTth dna polymerase: Recombinant thermostable DNA polymerase originally isolated from the bacterium *Thermus thermophilus*. rTth has optimal activity at 70°–80° and survives the denaturation steps of PCR. In addition to DNA polymerase activity, it has efficient reverse transcriptase activity in the presence of manganese.
Specificity: The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation proeducts, and matrix components.
Taq dna: Thermostable DNA polymerase that is originally isolated from the bacterium *Thermus aquaticus*, *Taq* has optimal activity at 70°–80° and is not degraded during the high-heat denaturation steps of PCR.
Template: A master copy used to start the DNA or RNA replication process.
Transcription: The synthesis of RNA using a DNA template.

APPENDICES

Appendix 1: Regulations and Standards

Nucleic acid-based techniques have rapidly transformed almost every field of research, pharmaceutical development, and diagnostics. The need for globally comparable, accurate, and reliable results in the diagnostic field has driven the development of national and international standards as well as fostered a highly developed regulatory environment. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT.[1] Virus-specific regulations and reference standards will be addressed in the Appendix to General Information chapter *Virology Test Methods* ⟨1237⟩. The following is a selective list of national guidance documents. For application-specific guidance the compendial user is referred back to the relevant regulatory agency for the most current guidance.

- FDA Center for Biologics Evaluation (CBER) "Review Criteria for Nucleic Acid Amplification-Based In Vitro Diagnostic Devices for Direct Detection of Infectious Microorganisms" (1993)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Biological In Vitro Diagnostic Product" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: In the Manufacture and Clinical Evaluation of In Vitro Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Viruses Types 1 and 2" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Use of Nucleic Acid Tests on Pooled and Individual Samples from Donations of Whole Blood and Blood Components (including Source Plasma and Source Leukocytes) to Adequately and Appropriately Reduce the Risk of Transmission of HIV-1 and HCV" (2004)

Appendix 2: Abbreviations

AABB	American Association of Blood Banks
ACD	acid citrate dextrose
ASO	allele-specific oligonucleotides
bDNA	branched DNA assay
BMA	bone marrow aspirate
CE-LIF	capillary electrophoresis and laser-induced fluorescence
CCD	charge-coupled device
cDNA	complementary DNA
CPR	cyclic probe reaction
CsCl	cesium chloride
Ct	cycle threshold
DEPC	diethylpyrocarbonate
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dNTP	dinucleotide triphosphate
DOP-PCR	degenerated oligonucleotide primed PCR

[1] Reference materials for nucleic acid-based techniques are available from National Institute of Standards and Technology (NIST), http://ts.nist.gov/measurement-services/referencematerials/index.cfm.

General Chapters

dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
dUTP	2′-deoxyuridine 5′-triphosphate
dTTP	2′-deoxythymidine 5′-triphosphate
ESI	electrospray ionization
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FEN	flap endonuclease
FISH	fluorescent in situ hybridization
FFPE	formalin-fixed paraffin embedded
FRET	fluorescence resonance energy transfer
GLP	good laboratory practice
HCV	hepatitis C virus
HIV	human immunodeficiency virus
ICH	International Conference on Harmonization
LAPS	light-addressable potentiometric sensor
LCR	ligase chain reaction
LED	light-emitting diode
LNA	locked nucleic acid
MALDI	matrix-assisted laser desorption–ionization
MDA	multiple-displacement amplification
MOPS	3-[*N*-morpholino]propanesulfonic acid
MS	mass spectrometry
mRNA	messenger RNA
NAT	nucleic acid amplification technologies
NASBA	nucleic acid sequence-based amplification
NTP	nucleotide triphosphate
OLA	oligonucleotide ligation assay
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	primer-extension-preamplification
PPi	pyrophosphate
QA	quality assurance
QC	quality control
RCA	rolling circle amplification
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
rTth	recombinant *Thermus thermophilus*
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
3SR	self-sustained sequence replication
SSCP	single-strand conformation polymorphism
STR	short tandem repeat
Taq	*Thermus aquaticus*
Tm	melting temperature; the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded
TMA	transcription-mediated amplification
TOF	time-of-flight
UNG	uracil-*N*-glycosylase
WGA	whole-genome amplification

⟨1126⟩ NUCLEIC ACID-BASED TECHNIQUES—EXTRACTION, DETECTION, AND SEQUENCING

NUCLEIC ACID EXTRACTION

Introduction

The basic principles of nucleic acid amplification technology (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. The current chapter covers general steps in the extraction and purification of nucleic acids from a variety of samples.

The expanding discipline of molecular biology in pharmaceutical and biomedical research and development is characterized by the rapid discovery of new markers for disease and technologies for their detection. Nucleic acid targets are isolated from a wide variety of specimens, and the quality and quantity of the extracted target are highly affected by specimen collection, handling, and choice of extraction procedure.

The analysis of complex organisms by molecular biological techniques requires the isolation of pure, high molecular weight genomic DNA and intact full-length RNA. The application of these techniques then allows the detection, identification, and characterization of the associated organism or adventitious agent. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay–Sachs disease, to name a few examples, or the analysis of single nucleotide polymorphisms (SNPs).

DNase and RNase are the major sources of nucleic acid instability. Although both enzymes are ubiquitous and are easily released during nucleic acid extraction, RNases are far more stable and harder to inactivate than are DNases because they generally do not require co-factors in order to function. Minute amounts of RNase are sufficient to destroy RNA, so great care should be taken to avoid inadvertently introducing these enzymes into the sample during or after the isolation procedure. If RNA is collected for the specific application of gene expression analysis, researchers should keep in mind that the sample collection process itself can alter the resulting expression profile.

Because of the ubiquity of RNases, measurement of intracellular RNA targets has lagged behind that of DNA targets in contributing to patient management and characterization of targets for pharmaceutical purposes. However, RNA represents the current status of the organism and is an important tool for correlating a phenotype with its associated genetic activity. The unstable nature of RNA has made standardization of NAT tests difficult, and false negative results can easily arise from a poorly handled sample because of target degradation rather than from the absence of disease or regulation of gene activity. Nevertheless, commercially available isolation and detection systems provide a high level of standardization and robustness, resulting in the implementation of RNA-based assays in recent years. The following sections discuss general steps in the extraction and purification of nucleic acids from a variety of samples, focusing on (1) collection, handling and storage of samples; (2) disruption of samples; (3) subsequent extraction and purification of nucleic acids; and (4) storage of purified nucleic acids.

Sample Source

The broad diversity of possible specimens requires different procedures for collection. For example, blood samples are collected in an appropriate anticoagulant- or additive-containing tube. Ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests that require plasma or bone marrow aspirate (BMA) samples. When extraction from tissues is appropriate, the optimal amount of tissue is usually 1 to 2 g, depending on the type of tissue, because the amount of DNA and RNA per weight of tissue varies greatly from tissue to tissue. In general, more than 10 mg of tissue is required to obtain >10 µg of DNA or RNA. Because of the highly variable amounts and types of proteins and other contaminants present in different tissues, nucleic acid isolation protocols are tissue-specific, and a broad range of ready-to-use isolation systems are available from different manufacturers of kits for nucleic acid extraction. The tissue type also influences the stability of both DNA and RNA in specimens, and the two types of nucleic acid differ importantly with respect to sample preparation and downstream analysis. These issues are described later in the chapter.

Pre-Analytical Steps and Sample Collection

Although the genetic makeup of the organism remains mostly unchanged over time, the mRNA population represents the current status of a cell under any given set of conditions, and thus is highly dynamic. To prevent degradation of mRNA and/or to preserve the original transcription pattern of the cellular mRNA, tissue should be placed immediately on ice or snap-frozen in liquid nitrogen. However, freezing disrupts the cellular structure and releases RNases. Hence, for RNA isolation in general (mRNA, ribosomal RNA, viral RNA, etc.), thawing in an RNase-inactivating buffer is essential. A more convenient procedure employs a stabilizing agent at ambient temperature. Several reagents for different types of sample material (e.g., tissue or bacteria) are commercially available. Vanadium salts were once used to inhibit RNase activity, but they have been superseded by

the use of chaotropic agents for the inhibition of RNase and stabilization of RNA. The sample can easily be collected in such reagents and stored for several days to weeks prior to RNA isolation.

For reliable gene-expression analysis, the immediate stabilization of the RNA expression pattern and of the RNA itself is an absolute prerequisite. Directly after the biological sample is harvested or extracted, changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as transcriptional induction. Such changes in the gene-expression pattern should be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

The use of gloves while handling reagents and RNA samples is mandatory to prevent RNase contamination arising from contact with the surface of the skin or from laboratory equipment. In order to create and maintain an RNase-free environment, laboratory personnel should treat water or buffer solutions with diethylpyrocarbonate (DEPC), which inactivates RNases by covalent chemical modification. Care should be taken because DEPC is irritating to the eyes, skin, and mucous membranes and is also a suspected carcinogen. Alternatively, commercially available RNase-free solutions and reagents may be used. Commercially available RNase inhibitor proteins are also available for use in reactions but with different levels of effectiveness with respect to various RNase types. However, it should be noted that DEPC cannot be used with Tris-buffered solutions. Many scientists recommend the use of disposable vessels when working with RNA. Nondisposable glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240° for 4 or more hours before use (autoclaving alone will not fully inactivate many RNases). Alternatively, glassware can also be treated with DEPC. Nondisposable plasticware should be thoroughly rinsed with 0.1 M sodium hydroxide and 1 mM EDTA, followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. The use of aerosol-resistant filter tips is also important for avoiding RNase contamination. These issues are not critical for DNA, and following the rules of Good Laboratory Practice (GLP) is generally sufficient for successful isolation of DNA.

As a general precaution, staff should follow all applicable safety precautions when handling tissue or body fluids (human or other). Some of these precautions (e.g., the use of disposable gloves) also prevent contamination of the sample. Applicable guidelines and standards for the collection and processing of human-derived materials have been published by the American Association of Blood Banks, the International Conference on Harmonization, and the FDA.

Sample Disruption and Homogenization

Prior to extraction, source material is disrupted and homogenized. Disruption is the complete breakage of cell walls and plasma membranes of solid tissues and cells in order to release all DNA and RNA contained in the specimen. This is usually done using a lysis buffer that also inactivates endogenous nucleases. In addition to disrupting tissues, homogenization shears high molecular weight DNA and cellular components. During RNA isolation, scientists often must reduce the viscosity of cell lysates (caused by the presence of high molecular weight DNA molecules) prior to final isolation in order to make the subsequent extraction steps easier and more efficient. Incomplete homogenization may interfere with subsequent RNA purification steps (e.g., inefficient binding of RNA to silica membranes) and therefore result in significantly reduced yields. A typical procedure to shear high molecular weight DNA and homogenize the sample is to repeatedly pass the lysate through a small-gauge needle. However, this procedure is time-consuming and is not suitable for high throughput of samples. Better procedures to achieve complete disruption and homogenization of cells and tissue include rapid agitation in the presence of beads and lysis buffer (bead milling) or rotor–stator homogenization.

During the bead milling process, disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the size and composition of the beads, the speed and configuration of the agitator, the ratio of buffer to beads, the disintegration time, and the amount of starting material. These parameters must be determined empirically for each application. For disruption with mortar and pestle, the samples should be frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. Standard safety precautions and the use of safety clothing to protect the skin and eyes should be employed when working with liquid nitrogen. Rotor–stator homogenizers are able to disrupt and homogenize animal and plant tissues within 5 to 90 seconds, depending on the sample. The rotor turns at very high speed, causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Other alternatives are commercial spin-column homogenizers in combination with silica-membrane technology, which provide a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples.

In order to achieve complete disruption, different sample types require different procedures. Cells from tissue culture grown as a monolayer or in suspension are easily disrupted by the addition of a lysis buffer that typically contains a mixture of an anionic detergent, a protease, and a chaotropic agent in a buffered salt solution. In contrast, nucleic acid isolation from fibrous tissues such as skeletal muscle, heart, and aorta can be difficult to disrupt because of the abundance of contractile proteins, connective tissue, and collagen. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using a lysis buffer and a proteinase.

In general, the same procedures are applicable for extraction of DNA and RNA. For DNA isolation more gentle procedures are preferable, but during RNA isolation, cells and tissues can be disrupted using a mixer mill because there is no risk of shearing the RNA. Certain downstream applications require high molecular weight DNA, and care should be taken not to shear the DNA molecules and thus render the DNA unsuitable for further analysis.

Extraction and Purification

Although several procedures are available for nucleic acid extraction, the suitability of a procedure depends on the starting material, the type and purity of nucleic acid isolated, and possibly the downstream application. The principal procedures are described below; several commercial kits are available to accommodate different sample types and applications.

PHASE EXTRACTION

The original technique for extraction of DNA and RNA from lysed samples is phase extraction, which involves nucleic acid extraction using a mixture of phenol and chloroform. Depending on pH and salt concentration, either DNA or RNA partitions in the aqueous phase. At neutral/basic pH, the DNA remains in the aqueous phase, and RNA remains in the organic phase or in the interphase (with the proteins). However, at acidic pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA, which remains charged, partitions in the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, followed by extraction with chloroform to remove any residual phenol. The nucleic acid is recovered from the aqueous phase by precipitation with alcohol. For RNA, this procedure is often combined with a protease digestion, alcohol or lithium chloride precipitation, and/or cesium chloride (CsCl) density gradients. A potential problem is contamination of the recovered DNA or RNA with organic solvents that may interfere with enzymatic downstream applications or spectrometry readouts.

CESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

For the isolation of high molecular weight genomic DNA, CsCl density gradient centrifugation is the traditional procedure. Cells are lysed using a detergent, and the DNA is isolated from the lysate by alcohol precipitation. The DNA is then mixed with CsCl and ethidium bromide and centrifuged for several hours at a high g force (typically 100,000 × g). The DNA band, which can be visualized under UV light as a result of the intercalation of the ethidium bromide with the DNA, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA. This procedure allows the isolation of high-quality DNA, but it is time consuming and also a safety concern because of the high quantity of EtBr involved.

ANION-EXCHANGE CHROMATOGRAPHY

An alternative procedure for the purification of high molecular weight genomic DNA is anion-exchange chromatography based on the interaction between the negatively charged phosphate groups of the nucleic acid and positively charged surface molecules on the anion-exchange resin. Binding occurs under low-salt conditions, and impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers. Pure DNA is eluted with a high-salt buffer and is desalted and concentrated by alcohol precipitation. This procedure yields DNA of a purity and biological activity equivalent to two rounds of purification in CsCl gradients, but in much less time. The procedure also avoids the use of toxic substances, and it can be adapted for different scales of purification. DNA up to 150 kilobases (kb) in length may be isolated using this procedure. Several kits are available for the isolation of DNA based on anion-exchange technology, and procedures vary in processing times and the quality and size of the isolated DNA.

SILICA TECHNOLOGY

The current procedure of choice for most applications is based on silica technology and can be used for isolation of full-length RNA or DNA with an average size of 20 to 50 kb. However, higher molecular weight DNA exceeding 100 kb is not efficiently extracted by this technology. The procedure relies on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts. Although both types of nucleic acid adsorb to silica, the use of specific buffers in the lysis procedure ensures that only the desired nucleic acid is adsorbed while other nucleic acids, cellular proteins, and metabolites remain in solution. The contaminants are washed away, and high-quality RNA or DNA is eluted from the silica using a low-salt buffer. The silica matrix can be used as particles in suspension, in the form of magnetic beads, or as a membrane. This technique is suitable for high throughput, and several kits and automated systems are commercially available. However, these aqueous lysis buffers (in contrast to lysis buffers based on an organic solvent such as phenol) are not ideally suited for difficult-to-lyse samples (e.g., fatty tissues). Kits designed to facilitate lysis of fatty tissues and to inhibit RNases are available. Silica-based kits provide a fast and reliable procedure for both DNA and RNA purification and are commonly used for nucleic acid extraction.

Although these procedures yield pure nucleic acids, for some applications in which even trace contaminations with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. Alternatively, procedures that use specific probe capture may be used. Relevant applications requiring such ultra-pure nucleic acids are discussed in *Nucleic Acid-Based Techniques—Amplification* 〈1127〉.

Specific Applications for Hard-to-Extract Materials

EXTRACTION FROM FORMALIN-FIXED AND PARAFFIN-EMBEDDED BIOPSIES

The nucleic acids in formalin-fixed paraffin embedded (FFPE) biopsies are usually heavily fragmented and chemically modified by formaldehyde. Although formaldehyde modification cannot be detected in standard quality control assays such as gel electrophoresis, formaldehyde modification does interfere with enzymatic analyses. Sufficient extraction and demodification for DNA can be achieved by prolonged digestion with protease, but this will lead to heavy fragmentation and degradation of RNA. Some isolation systems have been optimized to reverse as much formaldehyde modification as possible without further RNA degradation. Nevertheless, RNA purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR).

EXTRACTION FROM BACTERIA AND PATHOGENS

Although Gram-negative bacteria are relatively easy to lyse, Gram-positive bacteria or yeasts typically need an enzymatic pretreatment to remove the cell wall for efficient lysis. This methodology can be applied only to DNA isolation because the enzymatic treatment will influence the expression profile of the organism, and therefore RNA isolation requires a more rapid lysis procedure. Another factor to consider is that microorganisms normally occur against the background of a host or an environmental matrix (e.g., soil), which makes detection by polymerase chain reaction (PCR) often difficult because of inhibitory components. This means that the isolation procedure has to be carefully adapted and optimized for the specific organism and sample type. Commercial kits are available, and most are based on the use of lysozyme for the removal of cell walls.

SPECIAL CONSIDERATIONS FOR LIMITED SAMPLE AMOUNTS

Multiple genetic testing techniques, including SNP analysis, short tandem repeat analysis, sequencing or genotyping using arrays, real-time PCR, and other procedures depend on the availability of high-quality DNA. Because human genomic DNA or samples of individual genotypes are often limited, a process to immortalize nucleic acid samples can overcome this limitation. Procedures applicable to genotyping are discussed in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩. Whole-genome amplification (WGA) has recently been employed to amplify limited genomic DNA from already purified DNA or directly from clinical or casework samples without any DNA purification. Two basic technologies for WGA are available and are PCR-based or rely on isothermal multiple-displacement amplification. These applications are described in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

Sample Handling and Long-Term Storage

DNA is a relatively stable macromolecule, and once isolated it can be kept at 2° to 8° for at least 1 year. However, where DNA is present in very small quantities, such as in a test of residual DNA, it may be advisable to store the DNA at less than or equal to −20°. Generally, DNA is stored in solution. Distilled water can be used if DNA will be used for PCR and/or endonuclease digestion within a few days after its isolation. However, Tris–EDTA at pH 7.5–8.5 is the preferred buffer for DNA storage because DNA degradation can occur in water because of the limited buffering capacity of this medium. Purified nucleic acids retain recognizable characteristics during long-term storage, provided the samples are stored as frozen solutions. The DNA solution should be stored as a primary stock solution frozen at −80°. DNA can also be lyophilized and stored dry without the need for refrigeration. In some cases DNA can be stored for years on special filter papers that bind DNA and allow storage in a dried state at ambient temperature.

The ubiquity of RNases requires extra precautions when handling RNA. Isolated RNA should be kept on ice when aliquots are pipetted. Filter tips that prevent RNase carry-over from the pipette and sterile, disposable polypropylene tubes are recommended throughout the procedure because these tubes are generally RNase-free and do not require any pretreatment to inactivate RNases. Purified RNA can be stored at −20° or −80° in water. Under these conditions no degradation is normally detectable. Unlike DNA, RNA does not benefit from basic buffer solutions during long-term storage because of its sensitivity to alkaline conditions. Generally, if nucleic acid samples are required for multiple testing, RNA and DNA samples should be frozen in multiple aliquots at −80° for subsequent analysis in order to avoid repeated freeze–thaw cycles that can lead to degradation, and also to minimize the possibility of contamination, which could result in analytical inaccuracy.

QUALITATIVE AND QUANTITATIVE EVALUATION OF NUCLEIC ACIDS

Introduction

This section describes procedures that assess the purity, integrity, and quantity of purified nucleic acids, including spectroscopic procedures, electrophoresis of nucleic acid fragments, and probe-based techniques. Detection and quantitation by amplification are discussed in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩.

ABSORBANCE SPECTROSCOPY

The basic principles of spectroscopy are addressed in *Ultraviolet-Visible Spectroscopy* ⟨857⟩. For nucleic acids, absorbance is determined at 260 nm, but this procedure does not distinguish between DNA and RNA. Absorbance can also be used to estimate protein contamination in nucleic acids. Proteins maximally absorb at 280 nm, and nucleic acids maximally absorb at 260 nm. Thus the calculation of the A260/A280 ratio is used as an estimation of protein contamination in nucleic acid preparations. A ratio of 1.8 to 2.0 is considered desirable. As an example, double-stranded DNA has an extinction coefficient of 20 for 1 mg per mL of DNA at 260 nm and a coefficient of 10 at 280 nm. In contrast, for 1 mg per mL of protein, the extinction coefficients are on the order of 1 at 280 nm (depending on tyrosine and tryptophan content) and 0.57 at 260 nm. Thus a large protein contamination could exist at a 260/280 ratio of greater than 1.8 because of the lower sensitivity of protein absorbance. In addition, the change of absorbance of DNA with wavelength ($\Delta A/\Delta\lambda$) is steep at 280 nm, and this could lead to an incorrect determination if the spectrophotometer is out of calibration. The peak at 260 nm is broad, and thus readings are less sensitive to calibration issues.

Information on contamination by nonproteinaceous materials can be provided by a scan of DNA from 220 nm to 320 nm. Pure DNA has a mostly symmetric peak around 260 nm, zero absorbance at 320 nm, and a minimum at 230 nm. Absorbance rises again from 230 nm to 220 nm. Interfering substances can co-purify with DNA and absorb in the lower UV range (around 230 nm). These substances can interfere with and lead to an overestimation of DNA content, thus showing the utility of a scan—or at least a measurement of absorbance—at 230 nm in addition to 260 nm and 280 nm. Absorbance above 300 nm can arise from other contaminants and particulate matter. Common reagents used in the isolation of DNA, particularly solvents such as phenol and alcohols if they are not completely removed, can interfere with DNA absorbance measurements. Analysts should be aware of the limitations of this type of measurement. Finally, the absorbance of DNA and the 260/280 ratio is dependent on ionic strength—a difference as large as 30% can exist. Absorbance of genomic DNA is higher, and the 260/280 ratio is lower in pure water when compared with the same DNA in a buffer or a salt solution.

For the purposes of quantitation of nucleic acids, the respective extinction coefficients for DNA and RNA are used. An absorbance of 1 in a 1-cm cuvette corresponds to 50 µg per mL of double-stranded DNA [E (specific absorption coefficient) = 0.02 (µg per mL)$^{-1}$ cm^{-1}]. The specific absorption coefficient for RNA at 260 nm is E = 0.025 (µg per mL)$^{-1}$ cm^{-1} (absorbance of 1.0 corresponds to 40 µg per mL), and for single-stranded DNA E = 0.027 (absorbance of 1.0 corresponds to 37 µg per mL). A solution of DNA is read against a blank of the same buffer solution in which the DNA is dissolved. Ideally, readings should fall within a range of 0.1 to 1.0 absorbance for adequate linearity. Absorbance above 1.0 becomes increasingly nonlinear as the absorbance rises. The accuracy of readings below 0.1 (5 µg per mL DNA) depends on the quality and noise level of the spectrophotometer.

Fluorescence Protocols for DNA and RNA Quantitation

Cyanine dye derivatives are used for the quantitation of nucleic acids because they specifically interact with nucleic acids (DNA, RNA, and oligonucleotides) and fluoresce only upon binding. The exact mechanism of interaction is not always fully understood but may involve intercalation in double-stranded DNA and surface binding.

Measurements can be performed using a fluorometer or a plate reader. The sensitivity of fluorescence with these dyes is much higher than that of absorbance, which gives these dyes great utility when DNA concentration is low (down to 25 pg per mL). The dye must be protected from light to avoid photobleaching. Linearity is maintained over three to four orders of magnitude. Calf thymus and Lambda phage DNA are often used as calibrants to construct a standard curve. Some of these dyes have been optimized to bind double-stranded DNA or single-stranded RNA and oligonucleotides. A DNA-binding dye will also bind to single-stranded DNA and RNA but at low ionic strength, and the signal is about 10% or less than that seen with double-stranded DNA for an equivalent mass of material. Thus, this methodology is preferred for DNA measurements when no effort has been made to remove RNA from the preparation. Another fluorescent dye is available and is optimized for RNA measurements. Using two different concentrations of this dye, analysts can detect RNA in amounts as low as 1 ng per mL and as high as 1 µg per mL. The dye also fluoresces with DNA but does not display an equivalent ability to minimize binding by the use of particular conditions (e.g., with DNA and the double-strand binding dye). Quantitation may be affected by contaminating nucleic acid (e.g., DNA in an RNA preparation and vice versa). Treatment with a DNase is needed if DNA is present in the RNA preparation. Proteins are unlikely to interfere with these dyes, but some detergents as well as phenol result in loss of fluorescence. Nucleic acid extraction reagents should thus be checked for effect on subsequent fluorescent assays.

Bisbenzimide fluorochrome dyes such as (2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1*H*-benzimidazole) represent another option for measuring DNA. Researchers have studied the binding of these dyes to the minor groove of DNA and have found that sequences of adenine or thymine in the DNA sequence provide a minor groove dimension that binds the dyes best. Thus the fluorescent signal can show DNA sequence dependence, and the calibrant DNA should have a nucleotide composition that is similar to that of the DNA to be measured. These dyes are not as sensitive as cyanine dyes but are more sensitive than absorbance measurements. Low dye concentrations and high ionic strength are required in order for analysts to distinguish double-stranded DNA from RNA. Low ionic strength conditions are required in order to differentiate double-stranded DNA from single-stranded DNA.

Detection by Size

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis provides a simple and accurate procedure for separating nucleic acids by fragment size. The technique can be adapted to separate fragments over a large range of sizes and can be used in a preparative or analytical fashion. For example, gel electrophoresis can be used to verify that a product of a PCR reaction is of the correct size. DNA fragments can be retrieved from a gel slice and provide a sufficiently pure PCR product for cloning or sequencing. The general integrity of an RNA preparation can be determined by gel electrophoresis as well. The stoichiometry of the nucleic acid fragment size (in base pairs) and negative charge from the phosphate provide the basis for the separation. With the exception of plasmids, electrophoresis is generally free of DNA conformation-induced effects. Supercoiled plasmid DNA will migrate ahead of linear or open-circle/nicked plasmid, which is useful for determining the conformation of a plasmid preparation. In contrast, denaturing gels are used for RNA because of RNA's tendency to form inter- and intramolecular secondary structures.

Agarose gel electrophoresis utilizes a horizontal setup wherein the gel is cast in a box and placed on a bridge between two buffer compartments that are filled with the buffer of choice. The gel is also covered with a thin layer (~1 mm) of buffer. Although the main electrical resistance resides in the gel itself, there is sufficient charge on the nucleic acids to move fragments through the gel toward the anode. The fragments move in proportion to size, the smallest moving the fastest. The parameters that most affect electrophoresis are gel pore size, buffer concentration, and the voltage gradient. The ability to separate the fragments of choice is largely a function of the gel pore size, which depends on agarose concentration. Generally the agarose concentration is in the range of 0.5% to 1.0% for DNA fragments of <100 to 25,000 base pairs, and the higher concentration is used when it is important to distinguish the smallest fragments. Lowering the agarose concentration in the gel results in the resolution of larger fragments but also in a loss of resolution of small fragments. For the largest fragments pulsed, (reversed)-field electrophoresis is utilized.

To achieve uniform electrophoresis, all of the agarose must be completely melted. Electrophoresis-grade agarose is dissolved in the same buffer that will be used for electrophoresis. The buffers most commonly used for DNA separations are TBE (tris-borate-EDTA) or TAE (tris-acetate-EDTA). TBE has a higher buffering capacity than TAE, but TAE should be used if the DNA is going to be retrieved from the gel. Denaturing RNA gels use MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0). Melting the agarose is conveniently achieved with the assistance of a microwave oven. The agarose will easily come to a boil, but this may not result in complete melting of the agarose, which may require bringing the solution to a boil several times, with intermittent mixing and holding periods, until the agarose is completely melted. Agarose particles transform from white to transparent before melting. Any partially melted agarose can be detected by swirling the flask while holding it up to the light. If the solution does not appear uniform, then it requires additional heating. The agarose is poured into the gel box after partial cooling but before setting up. Commercially available ready-to-use gels suitable for a particular application can also be used. For RNA-denaturing gels, formaldehyde is added under a fume hood to the melted agarose to a final concentration of 2.2 M or 6.7%. Before the agarose has hardened, the analyst places a comb in the gel to provide wells for the samples and size standards. Once solidified, the gel is placed in the electrophoresis box, and buffer is added until both sides are filled and there is a layer of buffer across the surface of the gel. Then 10X tracking buffer (40% sucrose with 0.25% bromphenol blue or 0.25% xylene cyanol or both) is added to each DNA sample to increase the sample density and to provide a tracking dye that is used to assess when the electrophoresis is finished. The increased density allows the sample to be transferred into the well and to remain there until it migrates into the gel during electrophoresis.

One or more lanes should be used for a DNA size standard containing fragments in the range that is relevant to the samples and agarose concentration. Size standards in various ranges are readily available. Bracketing the samples in wells between standards is useful to determine whether the electrophoresis gradient has been uniform over the width of the gel. However, in the case of eukaryotic RNA preparations, the 18S and 28S ribosomal RNAs that are co-extracted from prominent bands (corresponding to 1900 and 4700 nucleotides) can also be used as size standards. In addition, the rRNA provides information on the RNA integrity because missing or fuzzy rRNA bands indicate problems with the quality of the RNA preparation. Once the wells are filled, the cover is placed over the gel box, and the box is connected to the power supply. The indicator dye in the tracking buffer added to the samples and size standard allows the easy determination of how far the electrophoresis has proceeded. Bromophenol blue will migrate with DNA fragments of <500 base pairs, and xylene cyanol will migrate with fragments of 5000 base pairs.

The power supply is frequently run under conditions of constant voltage (1 to 10 V per cm) of gel length. Elevated voltage can cause high current, resulting in the generation of damaging heat and exhaustion of the buffer.

PULSED-FIELD ELECTROPHORESIS

This variation on agarose gel electrophoresis is used to separate a range of large DNA fragments and is most useful when resolution of 50,000 to 200,000 base-pair fragments is needed. The main difference is the addition of an alternating-field device that controls the power supply operating under constant voltage. Large fragments of DNA change conformation in order to move through the agarose pores, and the larger pieces take longer to readjust when the field is reversed and thus move more slowly than do smaller fragments. This allows resolution of fragments over the period of hours that the pulsed-field procedure operates. A commonly used ratio of forward to reverse is 3:1, and, in addition, the procedure typically calls for a stepwise increase in the unit time between reverses of the field. Electrophoresis may continue for 10 to 16 hours to avoid fluctuation in gel temperature, viscosity, and other properties that may cause artifacts.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The format for performing PAGE is quite different from that for agarose gel electrophoresis. The general procedure for PAGE is described in *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* 〈1056〉. For resolution of small fragments of DNA in the 10 to 500 base-pair range, nondenaturing polyacrylamide gel electrophoresis is more suitable than agarose gel electrophoresis because separation of fragments of this size requires much smaller pore size than is achievable in agarose gels. The gel is prepared by polymerization of acrylamide monomers. The percentage of acrylamide dictates the range of fragment sizes that can be best resolved. For example, 20% acrylamide is suitable for the 10–100 base-pair range, and 5% acrylamide is useful in the 100–500 base-pair range. Commercially available ready-to-use polyacrylamide gels suitable for the particular size discrimination can also be used. The separated nucleic acids are visualized by staining with, for example, silver nitrate solution rather than with ethidium bromide or cyanine dye. However, staining with silver nitrate is laborious and time-consuming and not suitable for preparations that contain a large amount of protein, because proteins will also stain with silver nitrate.

CAPILLARY ELECTROPHORESIS AND LASER-INDUCED FLUORESCENCE (CE–LIF)

CEF has been used for many years to separate DNA fragments (for the general principles of CE, see *Capillary Electrophoresis* 〈1053〉). The procedure relies on a principle similar to that underlying agarose gel electrophoresis. CE can utilize the cross-linked buffer systems applied in gel electrophoresis, but the technique can also use polymer-containing solutions (e.g., polymethylcelluloses) that are designed to create pores that entangle proteins. These polymer solutions may be added to the capillary between injections, allowing a "fresh" gel prior to each run. In addition, capillaries can be used for more injections than are possible for polymerized gel-filled capillaries. The resolving power of the separation depends on the size of the pores, which is based on the composition of the gel. Kits are available to separate fragments into the desired size ranges. Fragment sizes outside the resolution window can possibly be separated, but the separation may not be reliable or reproducible when the gel capability is exceeded.

Fragments can be detected by a variety of mechanisms. Detection utilizing UV absorbance is possible, but the preferred and most common detection procedure is laser-induced fluorescence (LIF). Fluorescence offers improvements over UV detection in terms of selectivity and sensitivity. In addition, the detection limits for fluorescence are two to three orders of magnitude better than those for UV. Although DNA is intrinsically fluorescent, the background fluorescence and complex laser spectroscopy required preclude routine use. The most common way to label DNA is described in the section above on fluorescent protocols for RNA and DNA quantitation. This system is widely employed because of its simplicity (the dyes are added to the sample or into the reaction buffer) and effectiveness. The advantages of CE include speed of analysis, sensitivity using minimum sample volumes, and the potential for automation. These are achieved mainly by the inherent miniaturization of the gel. Automated systems allow robust analysis of the quality, quantity, and fragment size of both RNA and DNA. CE applications have been especially important for evaluating the integrity of RNA because of the instability and progressive degradation of RNA caused by ubiquitous RNases, and new technologies that compare the ratios of 28S and 18S are improving the capabilities of these procedures.

FILTER HYBRIDIZATION AND IN VITRO LABELING OF PROBES

Introduction

Hybridization techniques were used early in molecular biology to identify individual nucleic acids and to estimate the degree of similarity between species. Hybridization is widely used in the procedures described in this and other chapters to visualize and identify nucleic acid sequences (see *Nucleic Acid-Based Techniques—Amplification* 〈1127〉, *Nucleic Acid-Based Techniques—Genotyping* 〈1129〉, and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* 〈1130〉). With the advent of restriction endonuclease digestion of DNA and electrophoretic separation by molecular mass, hybridization using labeled probes provided a way to visualize the organization of genes within a specific genome.

The hybridization techniques described are dot and slot blotting, Northern blotting, Southern blotting, in situ hybridization, and fluorescent in situ hybridization (FISH). All these techniques rely on the use of nucleic acid probes. Probes are oligonucleo-

tides with specific DNA or RNA sequences that have been labeled with radioactive, fluorescent, chemiluminescent, chemical tags or enzymes (reporter molecules). Hybridized probes bind to complementary sequences on the target nucleic acids and are used to visualize and characterize targets, as described below.

Dot and Slot Blotting

Dot blotting is the simplest and quickest of the hybridization techniques. The nucleic acids are directly applied to a support membrane, which may be a nitrocellulose or nylon membrane, without prior separation of the nucleic acid species by agarose gel electrophoresis. The nucleic acids are spotted onto the filter using a micropipettor or an apparatus such as a dot blot or slot blot apparatus. This consists of a membrane frame with a membrane sandwiched in between the two pieces of the frame. The bottom frame plate is connected to a vacuum manifold, and the top piece of the frame has slots through which the nucleic acids are loaded. The samples are loaded under vacuum and pulled through the membrane by vacuum, with the nucleic acid binding to the membrane, and then the filter is air-dried. The nucleic acids are fixed to the filter either by heating to 80° for nitrocellulose membranes or by exposure to UV light for a predetermined time for nylon filters. Hybridization with a labeled probe provides confirmation of the identity of the nucleic acid but does not provide any information about the number or sizes of the species. The nucleic acid species of interest can be quantitated by spotting known concentrations of the purified nucleic acid on the filter and comparing the signal generated by the unknown samples with those of the standard preparations.

Southern Blotting

Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane. Small, single-stranded DNA probes can then be used to visualize and identify the DNA species of interest. Southern blot analysis is based on a transfer and immobilization methodology developed in 1975, coupled with the electrophoretic separation of fragmented DNA. More specifically, the procedure typically is used to identify specific nucleic acid sequences in the context of a defined genetic topography, such as a restriction endonuclease map. The position of genes within the viral genome can be accurately mapped using a variety of restriction endonucleases in combination with Southern blot analysis. The procedure requires that DNA be obtained in sufficient quantity for analysis. Fragmented DNA is separated according to size using agarose gel electrophoresis. Double-stranded DNA fragments must be denatured before they are transferred and immobilized on a membrane by capillary action. The immobilized DNA is then cross-linked to the filter, which may be composed of nitrocellulose or nylon, as described above. However, the use of positively charged nylon membranes eliminates the need to fix the DNA to the nylon membrane. Nitrocellulose membranes are more fragile and may be probed up to 3 times with separate probes. Nylon membranes are more robust and may be probed 10 to 12 times, but they may present more background noise, particularly when they are used with chromogenic probes.

Northern Blotting

Northern blot analysis comprises a series of steps for the separation, transfer, and immobilization of RNA in a manner similar to the treatment of DNA using Southern blot analysis. Denaturation of the RNA is required to reduce secondary structure to ensure that the RNA separates in the agarose uniformly according to length. Denaturation of RNA is accomplished either prior to electrophoresis using glyoxal or dimethyl sulfoxide (DMSO) or during electrophoresis by means of gels that contain formaldehyde. Transfer is achieved in a manner identical to that used for Southern blotting. However, in the case of Northern blotting, it is unnecessary to denature the RNA prior to transfer because denaturation is accomplished before electrophoretic separation of the RNA species. The immobilized RNA is cross-linked to the membrane in a manner similar to the cross-linking of DNA.

In Situ Hybridization and Fluorescent In Situ Hybridization (FISH)

Hybridization of a nucleic acid in situ classically refers to determining the location of that nucleic acid sequence in its natural state—in tissue, in individual cells, or on a chromosome. In situ hybridization probes are designed to bind to complementary nucleic acid sequences, whether they be DNA or RNA. The purpose of these hybridization procedures is to discover where in a tissue a gene is being expressed, in which case the target is RNA, or to map a specific DNA sequence to its location on a chromosome, in which case the target is DNA.

Chromosome mapping of DNA sequences is accomplished by chemically attaching silver grains to the probe sequences and then counting the density of the grains in a metaphase chromosome spread. Although, historically, these procedures worked well, sensitivity was always an issue. The solution was to use a reporter that was more sensitive and safer than the other reporters, namely, fluorescence used in the technique of fluorescent in situ hybridization (FISH). FISH has an additional benefit in that the different colors available in fluorescence afford the ability to observe multiple hybridization events simultaneously, a feature not available with other detection systems.

Detection of DNA and RNA in Hybridization Assays Using Labeled Probes

Visualization and location of individual nucleic acid species of interest are achieved by the specific hybridization of DNA or RNA probes that are labeled for easy visualization. The filter or sample (fixed cells or tissues in the cases of in situ hybridization and FISH) is incubated with the labeled probe at an appropriate temperature and salt concentration that allows hybridization of desired stringency. This is followed by washing with buffers of varying detergent and salt concentrations and at varying temperatures in order to minimize background signal due to nonspecific hybridization. The labeling and types of probes are discussed below.

Probes can be RNA probes generated in vitro or DNA probes, either double-stranded fragments, plasmids, or single-stranded oligonucleotides containing moieties to facilitate detection of fragments that contain portions of the gene of interest. Probes can be labeled with radioactive tracers such as ^{32}P or ^{35}S by incorporation of a labeled nucleotide in the probe sequence or with a nonradioactive label such as biotin by incorporation of a modified base, such as adenine monophosphate linked to biotin. Radioactive probes are visualized with X-ray film placed over the blot. Biotin-labeled probes are detected with a conjugate of streptavidin–alkaline phosphatase. An enzymatic reaction is run with alkaline phosphatase and a substrate that yields an insoluble colored product at the site of the probe. Variations on nonradioactive probes utilize other modifications to the DNA and linked antibody–alkaline phosphatase, as well as chemiluminescent probes that are detected on film.

Nucleic acids can be synthesized and manipulated by either enzymatic or chemical means. These same systems can be used to modify nucleic acid structure and to introduce foreign moieties to create unique molecules that can provide an advantage to the detection of limiting viral nucleic acids against a background of host nucleic acids. The chemical synthesis of nucleic acids and their purification has become routine, and high-quality synthesis and purification are commonly achieved. Moreover, larger segments can be synthesized, and when even larger segments are required, the subsections can be designed for concatenation and ligation.

Custom synthesis of DNA oligonucleotides is readily achievable in the laboratory using commercially available reagents and equipment. Alternatively, probes can be custom ordered from numerous commercial providers. Size-exclusion procedures for purification generally are used to eliminate incomplete oligonucleotides. RNA oligonucleotides also may be chemically synthesized or generated in vitro using complementary cloned DNA fragments under the control of various prokaryotic RNA polymerase promoter sequences. The use of DNA probes is much more common, but there may be some applications in which the increased association of RNA–RNA or RNA–DNA hybrids is advantageous.

The principal procedures of labeling DNA are direct labeling using a kinase reaction to attach a labeled nucleotide to the end of each DNA strand, by incorporating labeled nucleotides into a nicked DNA by utilizing the DNA repair function of the Klenow fragment of *Eschericia coli* DNA polymerase I enzyme (nick translation), and by PCR. This last procedure generates a relatively higher yield of internally labeled probe because each round of thermal cycling doubles the amount of labeled probe, whereas the former procedures result in a ratio of less than one probe molecule per template molecule. The PCR procedure also is used to generate unique probes with a variety of moieties located at the termini.

NUCLEIC ACID SEQUENCING

Introduction

The first DNA sequencing procedure, described in 1977, utilized chemical cleavage to specifically introduce chain breaks in a DNA sequence (Maxam and Gilbert sequencing). The procedure proved to be of significant utility in the early years of molecular biology, but it has not been used to perform high-volume sequencing and therefore is not discussed in detail here. The majority of sequencing performed today is based on the dideoxysequencing procedure, also described in 1977 (Sanger sequencing). This procedure fundamentally changed sequencing by exploiting the enzymatic specificity of polymerases that introduce strand interruptions at specific bases. This is the most widely recognized sequencing procedure and is considered a routine assay in molecular biology laboratories. Innovations in instrumentation, sample preparation and collection, data management, data analysis, and sequence assembly have relied on this sequencing procedure as their fundamental sequence generator.

High-throughput sequencing takes all the elements of the sequencing procedures and applies them to a mass collection of sequence data, typically for larger genomes, but high-throughput sequencing certainly may be used for smaller projects as well. Obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and data finishing. The technology to achieve these individual objectives includes the instrumentation, disposables, protocols, and procedures.

Sequencing Reaction

The dideoxysequencing procedure takes advantage of specificity of the Klenow enzyme to introduce chain-terminating nucleosides, called dideoxynucleotides, intermittently during the polymerase extension process. The sequencing of each sample requires four separate reactions (one for each base). The resulting mixture of various nucleotide chain lengths is then separated

on the basis of individual molecular masses. The incorporation of radioactively labeled nucleotides during the sequencing reaction permits the detection of the nucleotide chains.

Improvements in biotechnology have led to the discovery of more robust enzymes with high fidelity, improved stability, and other attributes that have led to longer reads and improved sequence fidelity. These improvements have made possible the introduction of cycle sequencing, which is now commonly used. The principle of the cycle sequencing procedure is a combination of Sanger sequencing and aspects of PCR amplification, whereby dideoxynucleotides are incorporated into the amplified DNA. Cycle sequencing leads to a higher concentration of labeled fragments covering a wider range of sizes than does Sanger sequencing, leading in turn to a higher read length.

Separation Procedures for DNA Sequencing Fragments

The previous sections of this chapter deal with the treatment of intact DNA and RNA molecules; the following sections address the challenges of separating the fragments that result from the sequencing reactions, notably slab gel sequencing and capillary electrophoresis. Subsequent sections address detection technologies and sequence integrity.

Slab Gel Sequencing

Polyacrylamide gel electrophoresis, frequently referred to as slab gel electrophoresis, was the first separation mechanism employed for the separation of DNA sequencing fragments. As described above, the electrophoretic separation of DNA fragments is driven by the size of the fragments in the reaction mixture. However, for slab gel sequencing the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. In addition to the polyacrylamide in the gel, a denaturant such as urea is frequently included to ensure denaturation of the fragments. Until the implementation of multicapillary sequencing systems, the separation power and throughput of slab gel separation mechanisms were often considered state of the art.

Capillary Electrophoresis Sequencing

As noted above, capillary electrophoresis offers significant advantages over gel-based separations. However, as with slab gel sequencing, the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. Multicapillary systems that utilize 8 to 384 capillaries are commercially available. These systems are the primary systems used for large-scale DNA sequencing, and, theoretically, they yield more than 1.1 billion base pairs of DNA sequences per year.

Detection

RADIOACTIVITY

The first detection strategies for DNA sequencing reactions utilized radioactive isotopes such as ^{32}P or ^{35}S, primarily because these were practical for gel separations. The advantages are that detection is universal, low limits of detection are possible, mobility shifts are eliminated, and fidelity differences for the DNA polymerases do not occur. Disadvantages include the high disposal and safety costs, the inability to multiplex (ultimately limiting throughput), and the need for 24 to 36 hours of exposure time (i.e., no real-time detection).

FLUORESCENCE

Fluorescence dyes have largely replaced radioactive isotopes as detection tools during DNA sequencing, mainly because they do not have the disadvantages of radioactive probes. Because the dyes can be discriminated by means of their emission maxima, multiplexing is possible, so four sequencing reactions per sample can be replaced by a single reaction using four different labels. Thus a single lane can be used rather than the four separate lanes that were necessary with radioactive probes. Additional advantages are higher throughput and automated data collection in real time.

MASS SPECTROMETRY

Mass spectrometry (MS) has revolutionized the field of biochemistry and has significant potential in the area of nucleic acid sequencing. Soft-ionization techniques such as electrospray ionization and matrix-assisted laser desorption–ionization have expanded the potential application of MS to DNA sequencing. MS offers some advantages over other detection methodologies, including speed of fragment detection (signal acquisition is in the range of microseconds versus hours for conventional approaches) and accuracy (e.g., the molecular mass of each fragment can be determined with a high degree of accuracy). The Sanger procedure makes use of mass differences of the fragments generated as part of the polymerization reaction. MS is sufficiently precise to resolve fragment sizes that differ by only one base pair. Unfortunately, the sensitivity of MS detection suffers as fragment length increases, and the 100-base-pair barrier has yet to be crossed.

More recently, other sequencing technologies have emerged that are based on massively parallel sequencing techniques that attempt to achieve low-cost sequencing. These techniques are based, for example, on solid-phase sequencing or they make use of highly parallel and miniaturized pyrosequencing, which is described in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩.

Sequence Integrity

A prerequisite for automated data collection and interpretation is that the data must be of good quality, which means minimizing human intervention and allowing the system to make base identifications following detection steps. It is a critical step to ensure accurate base identification by minimally sequencing both strands of the DNA several times. In addition, other tactics may be employed, such as using primers at different sequence positions, which can improve the accuracy of the developed consensus sequence. This task can be facilitated by the use of specialized software packages that are commercially available. More recent technology developments have produced alternative sequencing platforms that are more amenable to large-scale sequencing projects. These techniques include array-based platforms on which short stretches of target are sequenced on a chip that supplies raw data to sophisticated computational programs that reconstruct the sequence. Other sequencing approaches have been developed for the rapid sequencing of short nucleic acid sequences such as oligonucleotides of short PCR products. These technologies include MS-based and pyrosequencing platforms, the latter of which is described in *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩.

⟨1127⟩ NUCLEIC ACID-BASED TECHNIQUES—AMPLIFICATION

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid–Based Techniques—General* ⟨1125⟩. The current chapter covers major techniques that result in amplification of targeted nucleic acid sequences. The most common NAT assay is the polymerase chain reaction (PCR), which was first described by Kary Mullis. This procedure has been further refined to amplify a DNA fragment starting from RNA (reverse transcription-PCR, or RT-PCR). Initially, PCR was used in a qualitative manner to amplify and detect DNA molecules because its exquisite sensitivity paired with its high specificity made it a useful tool for the detection of nucleic acid targets. Since its inception, the number of PCR applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. In addition to the changes and improvements to the original design of the PCR procedure, alternatives to PCR are techniques used to amplify target nucleic acids to generate RNA instead of DNA amplicons. The most commonly used techniques are nucleic acid sequence–based amplification (NASBA) and the transcription-mediated amplification (TMA) which are described here in detail. In contrast to PCR, which relies on incubating the sample at three different temperatures, NASBA and TMA are based on isothermal conditions.

In addition to amplification of the target nucleic acid, the amplification step also can be directed at the signal used for detection (signal amplification). The most commonly employed technique is the branched DNA (bDNA) assay, in which the signal, typically a fluorescent probe that binds to the target sequence, is amplified. The bDNA assay is used predominantly for viral nucleic acid detection and quantitation.

This chapter describes the main assay components necessary for a PCR procedure and includes a discussion of the general optimization of PCR assays. The various PCR assay formats, including PCR, nested PCR, and RT-PCR are covered, and a discussion of the detection of the resulting amplicons follows. Although all these assays are essentially qualitative procedures, they can be modified for semiquantitation, and the various modifications are described. For accurate and reliable quantitation, real-time PCR has now replaced the methods listed above; real-time PCR and real-time RT-PCR are described in the *NAT Assays* section. The same section includes a discussion about probes and dyes that are an essential component of real-time PCR and the methods of quantitation using the generation of standard curves. The next PCR technique discussed is multiplex PCR, which is used for simultaneous detection of multiple targets or for normalization of assay results. Apart from PCR, the major alternative NAT tests that are used routinely, primarily in blood screening and clinical diagnostic screening are NASBA and TMA. The final technique described is whole-genome amplification, wherein the complexities of amplification require modifications to the PCR procedures. The chapter concludes with a discussion about the evolution of instrumentation used in NAT assays and the quality assurance and quality control issues associated with NAT because this is probably one of the most highly regulated biological techniques, especially when applied to blood screening.

ASSAY COMPONENTS

Enzymes

The essential components for NAT assays—polymerases, reaction buffers which include desoxynucleotides, ions, primers, probes, and fluorescent dyes—can be chosen from a broad selection of commercially available NAT reagent kits and vendors. Polymerases suitable for NAT applications can, in principle, be grouped into *Taq* DNA polymerases or DNA I polymerases from other *Thermus* species that are polymerases with features that are similar to those of *Taq* DNA polymerase. In addition, so-called proofreading polymerases are available (e.g., from *Pyrococcus* species) that display a 3′–5′ exonuclease activity capable of removing wrongly incorporated DNA bases from the growing DNA strand under amplification conditions. *Taq* DNA polymerase is the standard NAT enzyme and is the one most often used in NAT assays. Modifications of *Taq* DNA polymerase, such as deletions of the 5′–3′ exonuclease domain (Klenow fragment, Stoffel fragment) or point mutations for improved incorporation of dideoxynucleotides are also employed (e.g., for PCR-based sequencing reactions). Proofreading DNA polymerases or mixtures of *Taq* DNA polymerase with a proofreading polymerase are used if either fidelity of the NAT product is critical (e.g., for DNA cloning experiments) or longer NAT products are to be amplified. For RT–PCR, a reverse transcriptase is necessary to first convert the RNA target to copy DNA (cDNA) that can subsequently be amplified. For TMA reverse transcriptase with an RNAse H activity is needed to convert the RNA target to double-stranded template DNA, while for NASBA exogenous RHAse H is added to the reaction mixture. Depending on the reaction environment, two types of enzymes can be used to generate cDNA: a reverse transcriptase isolated from retroviral sources or a DNA polymerase that can function both as reverse transcriptase and DNA polymerase. Finally, chemical modification of the polymerase, resulting in an inactive enzyme at temperatures below 90°, is now typically used to prevent mispriming of templates at sub-optimal temperatures (see section on *Assay Optimization*).

Reaction Buffers

Reaction buffers vary with respect to ion composition, pH, and additives and are sometimes specifically adopted for particular applications such as multiplex PCR, real-time PCR, RT–PCR, TMA and NASBA. An important component of the reaction mixture is Mg^{2+} ions, or, in the case of polymerases with both reverse transcriptase and DNA polymerase functions, such as *Thermus thermophilus* (*Tth*), Mn^{2+} ions. Other additives that enhance the sensitivity and specificity of the assay may be present. The concentration of the four deoxynucleotide triphosphates (dNTPs) must be optimized.

Primers

Primer sets are oligonucleotides with sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Synthetic oligonucleotide primers for both standard PCR and for real-time or quantitative PCR are designed for the specific recognition of and binding to a single DNA or RNA sequence. Such specificity is achieved through design that involves both the length and the sequence of the primers. Length and sequence specifications have separate criteria that must be simultaneously met in order for the primers to perform properly. The length of a primer is a statistical issue that relates to the issue of the minimum length of a specific sequence necessary to guarantee that the desired target sequence is unique, regardless of the size or complexity of the genome. As an example, in the case of the human genome, with its 3.2 billion DNA bases, that length is 17 bases. For this reason the vast majority of PCR primers are between 20 and 25 bases long. The specificity of a primer should be determined by comparison with sequences in all known databases. Tools available on the Web facilitate such comparisons.

In terms of primer sequence, the issues to consider are T_m (the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded) and secondary structure. Every DNA has its own characteristic T_m, determined by length, sequence composition, and reaction environment. PCR primers are designed to bind to a perfectly complementary DNA sequence via guanine-cytosine (G-C) and adenine-thymine (A-T) base pairing. The T_m of the two PCR primers used in a reaction should be as close as possible. In terms of secondary structure, the formation of secondary structures by intra- or inter-complementarity should be minimized. Interaction between different primers can result in primer–dimers that will compromise assay sensitivity and specificity. All of the design issues presented are accounted for in any one of the dozens of primer design software packages that are available and can be found on the Internet.

Assay Optimization

NAT assay optimization is necessary for successful amplification that is sensitive and specific. Parameters that should be optimized include the thermocycling conditions, both temperatures and cycling times (that depend to a large extent on the target, primer, and probe sequences), concentrations of template, concentrations of NAT reagents, sample matrix and the number of amplification cycles. In the case of multiplex PCR, a compromise among elements of the reaction conditions is usually necessary because of the difficulties in optimizing the conditions for all the primer and probe sets. Recent changes have been made to improve sensitivity and specificity of NAT assays. One change is hot-start PCR, in which the addition of one of the

essential components of the NAT assay, typically the DNA polymerase, is temporarily withheld. When this occurs during reaction setup, the initial nucleic acid denaturation step prevents nonspecific amplification due to mispriming at suboptimal temperatures. Early hot-start procedures made use of wax barriers that effectively separated essential components into two liquid phases that were mixed only when the wax melted. However, this procedure has been replaced by two important hot-start technologies that do not require physical separation of the components by inconvenient additional handling steps. In the first procedure, antibodies directed against the DNA polymerase are complexed with the enzyme and lose their binding avidity at elevated temperature at the start of the reaction. The second procedure uses chemical modification of the polymerase, resulting in an inactive enzyme. At temperatures above 90°, typically in the first denaturation step, the modifier dissociates from the enzyme, and the enzymatic activity is restored. The advantage of an antibody-mediated hot start is the immediate release of enzyme activity at the start of the reaction by a very short heat incubation step. However, antibody-mediated hot-start chemistries tend to be less stringent when compared with chemically activated enzymes if there is a large excess of active polymerase molecules.

NAT ASSAYS

This section describes the basic techniques of PCR, nested PCR, and RT-PCR and procedural modifications that allow semi-quantitation.

Polymerase Chain Reaction

The PCR technique is based on a three-step process: denaturing double-stranded DNA (dsDNA) into single strands (ssDNA), annealing primers to the ssDNA, and enzymatic extension of primers that are complementary to the ssDNA templates. Each step is usually carried out at a different temperature. By cycling the temperature steps many times (usually 30 to 45 times), a billion-fold amplification of the target nucleic acid can be achieved, but the optimal number of cycles should be determined empirically. In some cases, especially where sensitivity is more important than false positive results due to excessive cycling, such as in blood screening, extra sensitivity can be gained by increasing the number of cycles to 60 to ensure that extremely low levels of target are detected. In a typical reaction, PCR product (amplicon) doubles at each cycle of amplification (exponential amplification). The increase in amplification in the early cycles follows a sigmoidal curve. In later cycles, the concentrations of the template strands and amplicons favor template strands re-annealing instead of PCR primer annealing to the template. At this point the concentration of the PCR product no longer doubles after each cycle, and the curve begins to plateau. A thermostable enzyme such as *Taq*-polymerase is a prerequisite because temperature cycling at 95° (the typical temperature step used to denature double-stranded templates) would inactivate a thermolabile polymerase.

NESTED PCR

An early variation of the PCR assay was nested PCR, which was designed to increase the assay's sensitivity and specificity. In this procedure amplicons from the initial PCR reaction are subjected to a second round of amplification using a different set of primers. This set of primers is specific to the amplicon sequence but is within the first set of primers (nested primers). The advantage of amplification with two sets of target-specific primers is increased specificity (any nonspecific amplification during the first amplification round would be reduced) and increased sensitivity (due to initial amplification of the target in the first amplification round). In addition, amplification of a product of the expected size is taken as confirmation of the presence of the target. However, a major drawback of this procedure is the high likelihood of cross-contamination due to the increased manipulation of amplicons generated in the first round of amplification. The use of highly specific primers and probes and the optimization of reaction conditions have resulted in the diminished applications of this procedure for routine testing, but the procedure is sometimes used for samples that are difficult to amplify by conventional PCR.

RT-PCR

In amplifying RNA targets, analysts prepare cDNA before the amplification step (RT-PCR). One-step and two-step RT-PCR procedures are available. In one-step RT-PCR the reverse transcription of RNA into cDNA and the subsequent amplification step are carried out in a single reaction without intermediate procedures. Therefore the reaction mixture for one-step RT-PCR includes the gene-specific amplification primers that are used for both reverse transcription and amplification. The advantage of this procedure is the overall reduction in handling time, increased throughput, and reduced contamination risk because re-opening the reaction vessel is not necessary. In contrast, in two-step RT-PCR the reverse transcription and amplification are performed as two separate steps. In general, random primers or oligo-d(T) primer rather than gene-specific primers are used for the reverse transcription step. An aliquot of the cDNA synthesis reaction is then transferred into the NAT reaction for subsequent amplification. The advantage of this procedure is the standardization of the reverse transcription reaction, which can be used as a single source for the analysis of multiple transcripts in gene expression analysis.

DETECTION OF AMPLICONS

Following amplification, analysts can employ a variety of procedures for detection of the amplicon as described in detail in the general information chapter, *Nucleic Acid–Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩. These include agarose gel electrophoresis with ethidium bromide or other dyes, capillary electrophoresis, and laser-induced fluorescence and hybridization followed by chromogenic detection such as streptavidin horseradish peroxidase detection, chemiluminescence, or fluorescent detection using labeled probes.

Quantitation—The original PCR and RT-PCR assays were qualitative and detected amplicons at the end of the reaction. Such detection is not easy to quantitate because at this stage the amplification is in a plateau phase at the end of the assay, and the amount of amplicon is not necessarily directly related to the quantity of the starting template. Several approaches have been deployed to attempt to overcome the shortcoming of PCR to produce reliable, quantitative results. Initial attempts at quantitation relied on assessing the amount of amplified DNA during the early or exponential part of the assay, but this procedure was fraught with problems because the aliquots had to be taken from the reaction mixtures at regular intervals, thus greatly increasing the risk of cross-contamination. One of the earliest and most straightforward approaches to quantifying PCR products was to measure the amount of amplicons that were generated during the exponential phase of the reaction by comparing this to a serially diluted external control. Several aspects, including variability in sample preparation and variations in reaction conditions, however, hampered this approach. Because of the exponential amplification of NAT procedures, even small errors or variances can lead to distinct differences.

Compared with dilution procedures, competitive PCR proved to be a much more precise approach to achieving reliable estimates of the originally present target molecules. This procedure relies on the simultaneous co-amplification of a specific target sequence in the presence of increasing concentrations of an exogenous target molecule (control) which shares the primer binding sites with the target sequence but whose sequence is slightly modified or shortened in order to facilitate discrimination from wild-type amplicons. In addition, the concentration of the control is known. The close sequence homology and similar size of the control and target amplicons are designed to ensure that the template and internal control are amplified with comparable efficiency. The relative strength of the amplicon bands of template and control can be assayed, for example, on ethidium bromide–stained agarose gels, giving a relatively precise quantitation of the wild-type target. A drawback of this approach is that the internal control and the template should be present in the reaction in approximately the same quantity in order to yield correct results. The development of real-time, quantitative PCR has eliminated the variability associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products.

REAL-TIME PCR AND REAL-TIME RT-PCR

Although gene quantitation by quantitative PCR was a widely used procedure, its applications were expanded by the advent of real-time PCR and real-time RT-PCR. Real-time PCR displays the same advantages as standard quantitative PCR—sensitivity, specificity, and a wide dynamic range—but the real-time procedure offers the additional advantage of requiring no post amplification processing because it combines amplification and detection in a single step. Real-time PCR collects data throughout the amplification process by measuring a fluorescence signal created as amplification progresses. A multitude of fluorescence chemistries allows the correlation of generated PCR product to fluorescence intensity. In principle, fluorescence intensity will increase with every cycle performed. Once the intensity is greater than background fluorescence, the so-called cycle threshold (C_t) value is achieved. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. The C_t value is inversely proportional to the number of target molecules in the sample and thus provides a means to quantitate the amount of target in the starting material (i.e., the greater the number of target molecules present, the lower the C_t value).

The reaction conditions for real-time PCR applications have to take into account the presence of the probe(s) and will require optimization. The most commonly used probes currently are hydrolysis probes, although hybridization probes are an alternative. In most cases, the amplification and detection steps can be combined into a two-step cycling reaction, but these conditions have to be optimized. In contrast, DNA-binding dyes which may also be used for amplicon detection require separation of the annealing and extension steps since the dye binding occurs during the extension step which is usually done at 72°.

A fluorescent DNA intercalating dye is used for detection of the PCR product in real-time mode. This dye emits light when bound to double-stranded DNA and the subsequent increase in fluorescence can be detected by real-time PCR instruments. Dyes that bind to dsDNA bind not only to the specific PCR product but also to artifacts such as nonspecific PCR products and primer–dimers. Analysts have observed substantial differences in the specificity of dsDNA-binding dyes in use with real-time PCR kits. Therefore, some analysts recommend verifying the presence of a single PCR product by gel electrophoresis to determine the correct size of the PCR product. Also, a melting curve analysis is advisable to ensure the absence of artifacts that could contribute to the fluorescent signal and thereby lead to misinterpretation of quantitative data. Alternatively, sequence-specific labeled probes can be employed. A wide variety of fluorescence-labeled probes and primers exist for use in real-time PCR and are described in the next section.

Real-Time PCR Probes—The difference between conventional PCR and real-time PCR is the presence of a third chemically synthesized oligonucleotide, the probe, which, for the most basic hybridization probes, contains some type of reporter molecule, usually a fluorescent molecule or fluorophore. Non-nucleic acid materials can be added to chemically synthesized DNAs

that are then incorporated into oligonucleotide probes for real-time PCR. Other applications include hybridization probes such as those used for fluorescence in situ hybridization (FISH) and microarrays and probes designed to capture other nucleic acids. A challenge arises in using fluorescent probes for real-time PCR because the unbound or free probe is not removed before detection, thus requiring a means to distinguish between signal obtained from bound and free probe. In contrast, FISH assays involve washing away free probe following hybridization.

All of the issues associated with primer design for conventional PCR apply to real-time PCR primers as well as to the probe sequence. As a general rule only two additional considerations apply to the probe sequence. One of these is thermodynamic, and the other specifically concerns the reporter moieties themselves. Thermodynamically, a good probe molecule that is designed to bind in the sequence somewhere between the two PCR primers will have a T_m that is about 5° higher than that of the two primers. In the large majority of cases the amplicon will be between 100 and 500 DNA bases in length, although for real-time PCR a smaller amplicon between 100 and 150 bases long results in a more efficient reaction. Thus it is rarely a problem to find a sequence inside a PCR amplicon that meets the necessary criteria.

Current probe designs overcome the problems of background from unbound probe using simple hybridization probes. In the original design, two probes that hybridize to adjacent sequences on the target nucleic acid are labeled. The reporter moiety is a fluorescent molecule attached to the 3′ end of the upstream probe sequence, and a second fluorescent molecule is attached to the 5′ end of the second probe. Excitation of the 5′ fluorophore with light energy of the proper wavelength results in absorption of that energy, followed by emission of light energy of a slightly longer or less energetic wavelength (Stoke's Law). This emitted energy then excites the 3′ fluorophore if it is close enough to the emitter and compatible with it in the sense that the emitted energy from the 5′ fluorophore can excite the 3′ fluorophore. When this occurs, the observed fluorescent light wavelength will be that of the acceptor molecule and not that of the donor. Fluorescence absorption and emission spectra are readily available for all of the commonly used fluorophores, and the only applicable rules are that the two fluorescent molecules must be fewer than 40 DNA bases apart and that the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Thus hybridization of the two probes, also known as hybridization probes or FRET probes (Fluorescence Resonance Energy Transfer), results in the emission of a fluorescent signal by the acceptor, and the latter signal can be detected. In the absence of hybridization, the probes are sufficiently separated in solution so that energy transfer cannot occur, and only background fluorescence is emitted by the donor.

Issues of fluorophore compatibility have been resolved by the increased use of a special class of molecule called a quencher. Quenchers are fluorescent molecules that absorb fluorescence energy over a wide range of wavelengths. Instead of re-emitting that energy as light they simply dissipate it as heat. Thus, if a quencher molecule is placed at the 3′ end of a probe and a fluorophore at the 5′ end, the probe will remain dark even when excitation energy is present so long as the molecule remains intact (hydrolysis probes). These probes utilize the 5′ nuclease activity of the DNA polymerase to hydrolyze a probe bound to its target amplicon. Cleavage results in separation of the reporter and quencher and permits fluorescence of the reporter. This reduces much of the work of optimization of the assay conditions (since only a single probe is used) and background noise generated with two probes.

A variation on hydrolysis probes involves placing the reporter and quencher molecules on a single oligonucleotide that is constructed so that, in the unbound state, the quencher and reporter are in close proximity, resulting in efficient quenching of the reporter. When the probe hybridizes to its complementary sequence on the amplicon, the probe undergoes a conformational change that forces the quencher and reporter apart, permitting fluorescence of the reporter. A variation on these kinds of probes is a combined primer and probe in which, again, the quencher and reporter are in close proximity in the native probe, thus resulting in no signal. Priming and subsequent elongation of the primer–probe results in hybridization to the newly synthesized DNA strand, causing spatial separation of the quencher and reporter and resulting in the generation of a signal.

Probe Labeling—Modern synthetic oligonucleotide modification chemistries permit the manufacture of oligonucleotides with non-nucleic acid materials. Placement of modifications is carried out in one of two ways: during synthesis or after synthesis. For the former, modifications are constructed in such a way that they behave like the four DNA or RNA bases that are routinely placed in the sequence. The modification is then presented in the desired location during the synthesis as if it were just another base in the series. In the latter, usually employed when more than one modification occurs, the synthesis contains a linker, such as an amino group, to which the desired modification is then attached. This process is often called "hand-tagging."

Perhaps the best-known example of hand-tagging is the conventional dual-labeled probe used in real-time PCR. The quencher is placed at the 3′ end of the sequence during synthesis, and the fluorescent reporter molecule is hand-tagged to an amino modification at the 5′ end of the sequence after the synthesis is finished and has undergone purification. Some modifications, such as biotins, are designed so that multiple modifications can be carried out in a single synthesis. Thus, it is possible to modify a synthetic DNA or RNA sequence to contain a number of different non-nucleic acid molecules. A cost is associated with such modifications insofar as alterations often are achieved with a loss of mass due either to an inherently lower efficiency of modifications to bind to the oligonucleotide as compared with standard DNA or RNA bases or to the requirement that the synthesis must be purified before modification, after modification, or both.

The benefits of modifying synthetic DNAs or RNAs usually outweigh the costs. The standard, quenched, dual-labeled, real-time PCR probe has permitted precise quantification of gene expression. Fluorescently labeled DNA oligonucleotides are also essential components of in situ hybridizations and microarrays. Some modifications confer increased thermal stability when synthetic DNAs or RNAs are hybridized to complementary DNAs or RNAs by comparison with unmodified DNA–DNA and DNA–RNA duplexes. These analogues include peptide nucleic acids, 2′-fluoro N3-P5′-phosphoramidates, and 1′, 5′-anhydro-

hexitol nucleic acids. Although such analogues succeed to varying degrees in achieving increased thermal stabilities, they fail to provide enhanced target recognition. Another approach is to use base analogues such as locked nucleic acid, which is an analogue that contains a 2′-O, 4′-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and stability.

The modification of a probe typically is governed by its intended use. Generally, fluorescent reporters are used in real-time PCR and for in situ hybridization. The range of available fluorescent reporters covers the spectrum from 517 nm to 778 nm. For hybridization probes, base modifications are preferred because these primarily alter thermodynamic interactions between bases, leading to improved specificity. Amino attachment groups, both with and without C-spacers, are used to attach other modifications to DNA sequences and to attach DNA sequences to solid surfaces such as glass slides. An example is the attachment of biotin molecules to DNA sequences. Biotin forms a strong bond with streptavidin-coated materials such as magnetic beads, allowing capture of specific nucleic acids that may themselves be hybridized to other molecules.

Quantitation—PCR products may be quantified using a standard curve drawn from replicate serial dilutions of a reference reagent or standard for the nucleic acid sequence of interest. The concentration of the nucleic acid in the reference reagent is known. Real-time PCR quantitation based on a standard curve may utilize plasmid DNA or other forms of DNA. However, the efficiency of PCR must be the same for the standards and the target samples. Performing PCR from purified targets can in some cases be more efficient than performing PCR with complex nucleic acid mixtures. The cycle threshold (Ct) values and concentrations of the dilutions of the reference reagent can be used to construct a standard curve from which the concentration of the unknown sample can be estimated. When the assay run conditions have been well standardized and the standard curve for a particular target has been well calibrated, in subsequent assay runs it may be sufficient to co-amplify only two dilutions of a reference reagent (usually dilutions containing known amounts of nucleic acid at high and low concentrations). These dilutions, or calibrators, can then be used to quantitate any unknown samples by comparison of the C_t values.

Multiplex PCR—Multiplex PCR describes the simultaneous amplification of several nucleic acid targets in a single assay reaction. This is a particularly demanding variation of PCR because it requires the use of a single set of reaction conditions for the amplification of multiple targets with different sequence characteristics. Additional complications can arise due to the increased chance of nonspecific amplification products arising from multiple primer interactions. In addition, the differing individual target amplification efficiencies can result in weaker reactions being out-competed by stronger, more efficient reactions.

Both qualitative and quantitative applications of multiplex PCR have been described in the literature, as have multiplex RT-PCR assays. Quantitative multiplex PCR relies on either the generation of multiple standard curves to enable quantitation of each target in the assay, or the inclusion of internal competitor sequences that can be used as calibrants.

Hybridization kinetics of primers and probes may be significantly different, even when designed using the same algorithm. This leaves the analyst with very limited room to optimize reaction conditions. However, optimization may include adjustment of DNA polymerase amount, Mg^{2+} to increase hybridization efficiency, or primer concentration. Especially in real-time PCR, optimization of primer concentration is critical for quantitative co-amplification of target genes. These are contained in the sample at significantly different amounts. Increasing hybridization efficiency of the primer–probe system can be achieved by providing sufficient reagents, such as Mg^{2+}, as well as adding a "molecular crowding" reagent that increases the effective concentration of all reaction components in the mixture. Multiplex PCR is not only used for genotyping applications, but also for quantitative real-time PCR because it offers several advantages over standard single real-time PCR reactions. Some of these advantages are a minimized amount of sample used, increased precision through the use of an internal control (e.g., housekeeping gene) co-amplified with the target gene in the same reaction, no separate pipetting steps, and cost-effectiveness.

Most PCR assays, however, suffer from a common problem—that of minimizing differences in extractions or amplifications between different samples. Multiplex PCR is useful in cases where it is critical to ensure that variability in quantitation of different samples is not due to differences in nucleic extraction or amplification measurements (usually when one measures the production of an mRNA species). Certain precautions and techniques can be employed to minimize these challenges; they are discussed in the next section on normalization of assay results.

Normalization of Assay Results—To minimize the effects of assay variables, analysts sometimes use a relative quantitation procedure that normalizes the target transcript level to a control that can be employed and compared for all samples included in the gene expression study. Probably the most reliable and most frequently used relative quantitation procedure relies on the measurement of "housekeeping" or control genes to normalize the expression of the target gene in a multiplex PCR format. This procedure is preferred because the quantitation of both the housekeeping gene and the target gene are influenced by varying cDNA synthesis efficiencies or the presence of enzyme inhibitors contained in the sample. However, it should be noted that the efficiency of conversion of target RNA to cDNA is not necessarily consistent even within a single-tube reaction but is a function of primer design, target sequence, etc. which may differ between target and housekeeping genes. The selection of appropriate control genes can cause problems because they may not necessarily be equally expressed across all unknown samples and may vary under experimental conditions. Normalizing measurements to a set of housekeeping genes in order to avoid the problem of variability may circumvent this concern. Alternatively, analysts can establish a thorough evaluation of housekeeping genes that do not alter gene expression levels under the experimental conditions.

All the NAT techniques described thus far are variations on the PCR assay, which is the most widely used of the NAT techniques. However, isothermal assays that are based primarily on the amplification of RNA are used for routine purposes. This is known as the transcription-mediated amplification (TMA) assay, which is closely related to the nucleic acid sequence–based amplification (NASBA) assay. Both assays are described in more detail in the following section.

NUCLEIC ACID CEQUENCE-BASED AMPLIFICATION AND TRANSCRIPTION-MEDIATED AMPLIFICATION

Both NASBA and TMA rely on in vitro isothermal amplification for detection and amplification of nucleic acids, also referred to as self-sustained sequence replication or 3SR. The major difference between the assays is that NASBA uses three enzymes—reverse transcriptase (RT), RNA polymerase, and RNase H—whereas TMA uses only two enzymes: RT and RNA polymerase. The complete procedure generally is performed at 41° to 42° using two primers. Both NASBA and TMA are especially suited to amplifying RNA analytes, including rRNA, mRNA, pathogens that have RNA as their genetic material, as well as DNA targets.

One of the primers that has a promoter sequence for the RNA polymerase at the 5′ end binds to the RNA target and is extended via the DNA polymerase activity of the RT. The product of this reaction is an RNA–DNA hybrid. RNase H activity then specifically digests the RNA strand of the hybrid, leaving only the cDNA to which the second primer can bind. A complementary strand of DNA is then synthesized by the RT, resulting in a dsDNA molecule with a T7 promoter at the 5′ end. The T7 RNA polymerase then transcribes multiple copies of the RNA amplicon. The RNA copies may undergo the same cycle to create new duplex DNA molecules with a T7 promoter from which many molecules of RNA are transcribed. Thus, unlike the action of PCR, the amplicon amplified in this case is of an RNA species.

Some of the characteristics of this technology are that only relatively short target sequences can be amplified efficiently (around 100–250 nucleotides); it uses a single temperature, which eliminates the need for special thermocycling equipment; the fidelity of the technique is comparable to that of other amplification processes; and the RNA amplicons are exponentially amplified. Carryover contamination is minimized because of the labile nature of the RNA amplicon in the laboratory environment. Containment procedures built into the assay procedure further help to minimize contamination. Detection of amplicons is typically achieved by the use of labeled probes and, in TMA technology, a common method is detection of chemiluminescent signals from hybridized probes that remain intact during the subsequent alkaline hydrolysis step used to destroy free probe.

The NAT techniques described, both PCR and TMA, are optimized for amplifying specific, small fragments of a genome. In cases when whole genome amplification is desirable, such as for mutation analysis or identity testing, modifications of the PCR procedure are necessary in order to ensure adequate sequence representation of genetic loci, as described in the following section.

WHOLE GENOME AMPLIFICATION

Historically, whole genome amplification (WGA) has been performed using modified PCR procedures. These procedures have relied on the nonspecific amplification of the genome using primers that bind under low-stringency conditions to the DNA template. PCR-based approaches differ mainly in terms of the type of primer employed in the reaction: in primer-extension-preamplification (PEP), short 15 base random primers are used in an initial cycling reaction at low stringency to make multiple random copies of segments of the genome. This product is then used as target for the specific PCR reaction. Amplification bias of favorable sequence contexts leading to uneven representation of the genome is the major drawback of this technique. The generation of increasingly shorter fragments during each round of amplification is a further drawback. Another procedure called degenerate oligonucleotide primed-PCR (DOP-PCR) uses tagged primers and low stringency amplification for the first few cycles of amplification followed by an increase in annealing stringency in later cycles. The tagged primers are characterized by defined sequence tags at the 3′ and 5′ ends and a random sequence in the centre of the primer. Under the later, more stringent conditions, the target DNA fragments generated during the first cycles containing the amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA typically employs *Taq*-like polymerases that possess the disadvantage of introducing variations into the amplified DNA due to their relatively low processivity and fidelity which become compounded by the very high number of amplification cycles used in these methods. This may cause problems in downstream applications such as genotyping analysis. These limitations as well as the relatively poor sequence representation of genomic loci inherent to PCR-based WGA can be overcome by an isothermal reaction called multiple displacement amplification (MDA).

The enzyme that is used for MDA comprises a high processivity polymerase with proofreading and strand-displacement activity. The isothermal reaction is performed at 30° without any change in reaction temperature. The reaction starts with the annealing of multiple random primers to the target DNA and elongation of the primers using a DNA polymerase from the *Bacillus subtilis* phage Phi29. Because the polymerase is able to displace DNA strands in a 5′–3′ direction, the polymerase reaction is not stopped when the elongating strands meet downstream DNA strands. The displaced DNA strand serves again as a target for multiple primed elongation reactions so that the DNA template is amplified exponentially in a branched-like manner, yielding high molecular weight DNA with a good representation of the genomic loci. Compared with PCR-based WGA, the error rate is very low. In particular, the mutation rate of repetitive sequence structures is low because of the limited strand-displacement activity of Phi29-polymerase. This permits reliable genotyping of genomic DNA (e.g., SNP analysis, mutation analysis, identity testing, or analysis of case work samples) on different platforms such as real-time PCR or array analysis.

General Chapters

INSTRUMENTATION

The development of the numerous and varied NAT techniques described in this chapter has been facilitated by the evolution of instrumentation that has served to automate these complex procedures. A general description of the major changes in instrumentation is discussed in this section.

The continuous control of the temperature steps necessary to achieve exponential amplification for PCR assays is carried out by fully automated thermocyclers that consist of a heating block in which the temperature can be rapidly cycled. Temperature changes are induced by water, or more recently, by using the Peltier effect. These instruments may be coupled to a fluorometer apparatus if they are used for real-time PCR analysis. In the latter case certain instruments are equipped with a rotor device that is heated and cooled by air instead of a metal block that typically is used as a heating module. In the case of endpoint PCR, PCR products are usually analyzed according to size on agarose or polyacrylamide gels, or by capillary electrophoresis using fluorophore-labeled primers. They may also be analyzed by an array-based approach or other hybridization procedures.

Because no post-PCR processing or label-separation steps are required, real-time PCR assays are simple to perform, making them useful for high-throughput applications. Real-time PCR instruments combine the properties of a thermocycler and a fluorometer to allow determination of PCR products by fluorescence measurement. In each PCR cycle, either one or several fluorescence readouts are taken to monitor the PCR reaction for generation of amplicons, usually at the extension step of the PCR reaction.

Real-time PCR instruments vary with regard to simultaneous sample throughput (32–384 reaction vessels), sample volume (5–100 µL), excitation source, and detector used. These compositions define the suitable range of fluorescent dyes for multiplex real-time PCR as well as size and heating/cooling principle (see above). The excitation source of real-time thermocyclers is either a laser-based system, halogen bulbs, or light-emitting diodes (LED). Optical filters are used to select the wavelength of interest. In most instruments, the emitted light is detected by a charge-coupled device (CCD) that consists of an array of light-sensitive cells. Light projected onto the CCD is converted to an electric charge, resulting in a signal that is proportional to the light intensity.

The versatility of the PCR assay has resulted in the widespread and diverse use of this technique. With the advent of real-time PCR, it has been possible to design high-throughput instrumentation for automated testing. Similarly, the TMA assay has also been automated. Such technology is used by laboratories doing high-throughput, highly regulated testing, typically blood screening for hepatitis C virus (HCV) or human immunodeficiency virus-1 (HIV-1) because automated tests are ideal in a regulated environment where minimum human intervention is required. The use of NAT in a highly regulated environment has resulted in the development of guidances for managing the quality assurance (QA) and quality control (QC) aspects of testing, as well as the validation of systems and assays as described in the following section.

QUALITY ASSURANCE AND QUALITY CONTROL FOR NAT

This section serves as a general guidance for the development of laboratory- and procedure-specific QC and QA procedures for NAT. Aspects such as waste management, management of radioactive material, or working with hazardous material are not covered. NAT is a technology that offers extreme sensitivity with its ability to generate millions of amplicons from as little as a single nucleic acid template, resulting in a detectable signal. The advantages of this technology can be offset by the necessity of establishing complex assay protocols and the requirement to follow carefully very stringent QC/QA protocols. Deviation from these protocols can cause major problems, such as false positive results due to the contamination of templates by amplicons generated in previous assay runs. Similarly, failure to control inhibitors could lead to suboptimal amplification and possible false negative results. Given the myriad factors that can greatly influence the outcome of a NAT assay, all aspects concerning NAT need to be covered by appropriate and stringent QC/QA procedures. This requires careful facility design, workflow, and selection of equipment suitable to the purpose. Data recording, record keeping, and data interpretation are other aspects that should be covered by QC/QA. Thus, QA for NAT assays includes assay validation, establishment of acceptance criteria and specifications, and adherence to good manufacturing/laboratory practices. These aspects are also described in this section. In addition, reference should be made to other published guidelines such as the ICH Guideline *Validation of Analytical Methods: Methodology* (Q2B) and the NCCLS Guidelines.

Laboratory QC/QA

An NAT laboratory should be designed and operated in a manner that prevents contamination of reactions with products from previous amplifications (carry-over) as well as cross-contamination between samples. Historically, the application of PCR required strict separation of the various steps of the assay in order to prevent cross-contamination of PCR by amplicons. This was necessary because early procedures for analysis of PCR products involved the transfer of the product, which potentially could lead to contamination. Therefore, in an open system the best measure to prevent contamination has been the strict separation of working areas for individual process steps. This includes individual areas for template preparation, master mix setup, distribution of the master mix to individual reaction wells and addition of template, space for cycling the PCR assays and, optionally, a separate work space for PCR product analysis. These requirements are not necessary with closed systems. With both open and closed systems it is still necessary to take additional precautions. These safety measures include UV illumination of work spaces overnight to inactivate residual DNA by crosslinking. In case of contamination, laboratory benches and pipettes

can be decontaminated by cleaning with a 10% solution of commercial bleach, which usually contains about 5% sodium hypochlorite, taking appropriate safety measures such as wearing gloves and eye protection. Afterwards, benches and pipettes should be rinsed with distilled water. A unidirectional workflow will reduce the opportunity for contamination to occur. Also, no materials, supplies, or equipment should be exchanged between designated working areas or rooms.

Equipment QC/QA

Other good laboratory practices that are related to the prevention of carry-over contamination include the use of suitable and clean equipment. Generally, disposable consumables (tubes, pipette tips, etc.) are highly preferable to reusable equipment. The use of disposable tips containing hydrophobic filters is another very effective measure to minimize cross-contamination. All samples, primer, probes, etc. must be labeled with relevant information such as identity of the content, date of use or preparation, expiration date, concentration, and storage information. Dedicated laboratory coats or disposable lab coats should be available in each room (or section) of the NAT laboratory. Appropriate gloves should be used during all processing steps to prevent sample contamination. The gloves should be changed frequently. Because heat sterilization does not completely destroy DNA, PCR products may lead to detectable contamination of, for example, glass surfaces. Following unique sterilization procedures for different materials such as waste and glass laboratory equipment is advisable.

Carry-Over Prevention with Uracil-*N*-Glycosylase

Contamination by PCR product carry-over can be mitigated by using the commercially available uracil-*N*-glycosylase (UNG) procedure. The procedure involves substituting 2′-deoxyuridine 5′-triphosphate (dUTP) for 2′-deoxythymidine 5′-triphosphate (dTTP) in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification, which can be easily incorporated as a first step into PCR cycling programs. Incorporating dUTP into the amplicon makes the PCR products biochemically distinct from the native DNA template. The enzyme UNG cleaves the deoxyuridine-containing PCR products by opening the deoxyribose ring at the C1 position. When the deoxyuridine-containing DNA is heated during the first thermal cycle, the amplicon DNA chain breaks at the position of the deoxyuridine at the alkaline pH of the PCR reaction mixture and thereby renders the carried-over PCR product nonamplifiable. Thus, any previously generated U-containing amplicon that might have contaminated another sample will become nonamplifiable. As a consequence, false positive results can be avoided. However, it should be noted that UNG has concentration limits above which it does not fully remove PCR carry-over products.

VALIDATION OF NAT SYSTEMS

Assay validation is achieved by

(1) ensuring the quality and consistency of assay components, including primers, probes, and enzymes; (including shelf life and contamination control) and

(2) establishing the performance characteristics of the NAT assay in terms of reproducibility, accuracy, ruggedness, robustness, specificity, precision, and analytical and clinical sensitivity.

The analytical sensitivity of an assay is defined as the minimum concentration of a reference reagent or standard detected by the test while the clinical sensitivity of a test is determined by testing clinical specimens and determining the 95% LOD. The clinical sensitivity of a test is not necessarily the same as the analytical sensitivity. The closer the reference or standard material is to the samples being tested the closer the correlation.

The principal steps of assay validation are

(1) sample preparation;
(2) consistent production of critical reagents;
(3) use of controls, calibrators, and quantitation standards;
(4) specimen and reagent stability;
(5) functionality of instruments and software;
(6) operator training; and
(7) laboratory surveillance for proficiency.

Following assay validation, further QA is necessary to monitor specifications and functional characteristics that have been established by the use of well-characterized reagents of known potency.

Quality Control of Reagents

DNA TEMPLATES

The test specimens used are usually, but not limited to, whole blood, plasma, and serum. Specimen preparation is a key step in the NAT assay and has a major influence on the performance and variability of the assay. Specimen collection is the first step in sample preparation. QC/QA staff should carefully evaluate the effects on the integrity of DNA of collection tubes and temperatures during sample transport. To prevent cross-contamination during specimen collection, aseptic techniques should be

used along with closed sampling systems in order to avoid specimen contamination. The use of appropriate sample handling techniques, temperature conditions, and anticoagulants or preservatives should help reduce the risk of contamination. Anticoagulants such as heparin or EDTA may interfere with the NAT assay.

SAMPLE EXTRACTION

The buffers, reagents, and detergent or chaotropic agents used for nucleic acid extraction should be evaluated for inhibitory effects on the NAT assay. Extraction controls, including spiked materials, should be included to monitor the efficiency and reproducibility of the extraction method. Reproducibility of the sample preparation method should be determined under the specimen processing conditions, including sample handling, storage, and shipping conditions. DNA is generally stable, but personnel should take care to avoid storage at refrigerated temperatures for extended periods of time to avoid sample degradation. Repeated freeze–thaw cycles can sometimes cause DNA fragmentation. In the case that the target is RNA, it should be noted that RNA is very unstable and specimens should be frozen.

PRIMERS

Primers and probes should be qualified in terms of purity, identity, and functional potency. Purity can be assessed by use of HPLC or mass spectrometry; identity can be established by sequencing; and functionality can be established by the use of reference reagents. However, in many cases, these methods may not be available for in-house testing. In these cases, it may be sufficient to compare lot-to-lot variation of purity and functional potency using relevant methods available in-house coupled with the use of reference reagents.

DNA POLYMERASES

The functionality of enzymes should be determined using reference materials. Enzyme preparations should be tested for other enzymatic activities; for example, exonucleases and DNA- and RNA-dependent polymerase activities and specifications should be established. Lot-to-lot comparison, as well as comparison with the manufacturer's CoA should also be done. Storage conditions recommended by the manufacturer should be strictly followed, and appropriate controls should be used to monitor the stability of enzymes.

Run Controls

The use of controls affords the operator assurance that the assay has performed within accepted specifications. In PCR testing, several steps in the testing process, as outlined above, should be monitored and verified. Multiple controls or controls that serve multiple purposes may be needed for a PCR assay. Controls should reflect the specific technology under development but should typically allow monitoring of ultracentrifugation, extraction, amplification, hybridization, quantitation, contamination, etc. Controls should be similar to the specimen type whenever feasible, although spiked controls may be acceptable.

A negative control is one that does not contain the target sequence or pathogen that is being tested. It should resemble as closely as possible the sample matrix under testing. Multiple negative controls should be examined, including nontarget sequences and nucleic acid-free controls to monitor for false positives resulting from contamination. Because of the high sensitivity of amplification assays, QC/QA personnel highly recommend that sponsors include control measures for the prevention of contamination events.

A positive control is one that contains the target sequence of interest. It should resemble as closely as possible the specimen matrix being tested and should contain an appropriate and defined amount of target sequences. (e.g., kit control).

Specifications for both positive and negative controls should be provided, as well as validation data supporting the proposed assay cut-off/reporting threshold value or the assay's limit of detection. The laboratory should define the source of the controls and calibrators and have a plan for their continued renewal. Controls can be infectious or non-infectious. In the latter case, validation of viral inactivation should be provided.

Reagent controls are often referred to as blanks and could include samples that have no target sequence, no enzyme, no primers, etc. These controls provide additional information about problems encountered in PCR assays.

An internal control is added to each specimen to ensure the overall validity of the individual test results. Internal controls are used to verify sample extraction, amplification, and detection.

External Quality Assessment and Proficiency Testing

Quality assessment of the laboratory is achieved by participation in periodic competency assessment and laboratory proficiency programs. The latter should include the testing of reference reagents and well-characterized panels to measure the technical proficiency of operators. Therefore, care should be taken to prevent cross-contamination, to monitor workflow, and to ensure careful specimen and test sample handling. Evaluation of operator proficiency should include participation in compe-

tency and quality assessment programs. Each operator in a particular laboratory should participate in such programs and should demonstrate comparable results.

Data Management

Complete and consistent documentation of all activities performed and all data generated is necessary. Such documentation does not only require the maintenance of records of the data generated through sample testing but also information about reagents and equipment calibration and maintenance. Moreover, any alteration in the assay procedure needs to be introduced through a planned change control process and documented in such a way that change can be assessed by an independent party.

⟨1128⟩ NUCLEIC ACID-BASED TECHNIQUES—MICROARRAY

INTRODUCTION

Microarrays are microscopic spots of DNA (measured in micrometers) arranged in an ordered manner (columns and rows) on a planar surface so that each DNA spot can be uniquely identified to facilitate an accurate analysis of the data. The DNA spots, also called array elements, are specific DNA molecules of known or unknown sequences and can be of similar or different nucleotide lengths. Samples of these mixtures are placed in fixed locations on the microarray.

Unlike conventional probes, which are a specific DNA or RNA sequence labeled with radioactive, fluorescent, or chemiluminescent tags (see *Nucleic Acid-Based Techniques—General* ⟨1125⟩, *Glossary*), the array elements are referred to as probes when the sequence information of the array elements is known, despite not being labeled. In this context, the target refers to labeled nucleic acids in solutions that are hybridized to the array elements or probes. The purpose of a microarray experiment is to identify the sequence of these labeled nucleic acids and/or determine their content. Compendial applications at this time are limited but may increase with wider use of microarrays in diagnostics and in drug discovery, development, registration, and control applications. When used for compendial purposes, standard assay development and validation approaches with availability of suitable reference materials are likely to apply.

Microarrays can range from hundreds to thousands of array elements (low density), tens to hundreds of thousands of array elements (high density), to millions of array elements (very high density). In addition to the use of planar surfaces for microarrays, the array elements can also be immobilized on individual support particles, such as beads. In these cases the array elements are identified by the particles themselves rather than specific locations on an array. The advantages of using microscopic spots on the array include high density, fast hybridization kinetics, and low sample volumes. Microarrays greatly speed up the acquisition of data, and in some cases increase the predictive power of results, by comparison with conventional nucleic acid-based assays. This is achieved by miniaturization, multiplexing, and parallel execution of nucleic acid-based tests that traditionally are performed in tubes, plates, or capillaries as described in general chapter ⟨1125⟩ (see also *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩, and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* ⟨1130⟩).

The principle of microarray analysis is the specific binding of the target DNA molecules to the probes or array elements. The ordered array of rows and columns of spots allows highly automated detection and analysis. DNA microarrays are manufactured, processed, detected, and analyzed in a number of different ways and have many applications. With the aid of computers, laboratory automation, and high-resolution detection devices, microarrays produce large amounts of data and are the analytical tool of choice to unravel the molecular complexity of DNA or expressed RNA.

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are described in chapter ⟨1127⟩. The present chapter covers the general field of microarrays, but detailed treatment of various application-specific microarrays, including data analysis and validation, are excluded from this chapter at this time. The following sections address the major applications of microarrays, sample processing, labeling, workflow, detection, and analysis of data. Several of these sections, for example, sample preparation and labeling, overlap with chapters ⟨1126⟩ and ⟨1127⟩, and cross references are made accordingly. Finally, regulatory aspects of microarrays will be discussed.

GENERAL PRINCIPLES OF MICROARRAY EXPERIMENTS

Types and Applications

Microarrays are most widely used in three types of analysis: gene expression, microarray-based comparative genomic hybridization (or array comparative genome hybridization, aCGH), and single nucleotide polymorphism (SNP). In brief, gene expression microarrays generally measure messenger RNA in a cell; aCGH analyzes DNA copy number variations, chromosomal

additions, and deletions in genomic DNA; and SNP microarrays are used in genotyping to analyze single nucleotide polymorphisms (see ⟨1129⟩). Within each type of microarray, various platforms, both manual and with various levels of automation, are available. *Table 1* summarizes the three major types and most common applications, as well as the target for each application, the probe, and the complementary nucleic acid techniques (see ⟨1126⟩, ⟨1127⟩, and ⟨1129⟩).

Table 1. Major Types and Applications of Microarrays

Types	Application	Target	Probe	Complementary Technology
Gene Expression	Gene Expression	mRNA	Oligonucleotide/cDNA	qRT/PCR, Northern Blotting
aCGH	aCGH CNV	DNA	Oligonucleotide/ cDNA/Pac, Yac, Bac	Cytogenetic chromosome analysis
SNP	SNP Genotyping	DNA	Oligonucleotides	Sequencing
	SNP	Amplicons	Oligonucleotide	Sequencing
	SNP	Oligonucleotide	Amplicon	Sequencing

GENE EXPRESSION MICROARRAYS

Gene expression microarrays are used to measure the relative level at which a certain gene is expressed. They are a powerful tool for target gene discovery, molecular tumor characterization, diagnosis, classification, treatment, and monitoring of diseases. Underlying molecular subgroups that are active in diseases have been identified by observing distinct and recurring gene expression subsets found within diseased tissues. Gene expression microarrays are also used to measure changes in gene expression over a given period of time, e.g., within various stages of a cell cycle or by identification of gene mutation(s) that lead to cancerous growth. Another application for gene expression microarrays is the development of new drugs, e.g. by measuring the down-regulation of a gene associated with a particular disease to monitor the effectiveness of a new drug. When the expression levels from a set of genes are measured, the term gene expression signature (biomarker or classifier) is used. Other examples of biomarkers or classifiers are drug activity classifiers that are used to diagnose the mechanism of action of a drug or toxicity classifiers that are used to diagnose and develop dosage parameters for a patient.

ACGH MICROARRAYS

In contrast to gene expression microarrays, aCGH microarrays target segments of DNA rather than individual genes (this is, similar to chromosomal banding and traditional comparative genomic hybridization). In an aCGH microarray, the array elements, which are large pieces of genomic DNA or specially designed oligonucleotides, are used to identify a known chromosomal location or changes. The primary advantage of aCGH is the ability to detect DNA copy changes at multiple loci in a single assay and to do so at a much greater resolution compared to traditional CGH. Depending on their design, aCGH microarrays provide distinct advantages over conventional cytogenetic analysis such as karyotyping and fluorescence in situ hybridization (FISH) because they have the potential to detect the majority of microscopic and submicroscopic chromosomal abnormalities. Compared to aCGH, these conventional cytogenetic techniques have low throughput, are labor-intensive, and often require specially trained staff to perform tests in a consistent manner. aCGH microarrays are also useful for the detection of cancer by monitoring the loci of oncogenes and tumor suppressor genes.

SNP MICROARRAYS

SNP microarrays identify the presence of known sequence polymorphisms by analysis of the pattern of hybridization to a series of probes that are specifically complementary either to wild-type or mutant sequences. If the SNP or set of SNPs associated with a particular disease are known, SNP microarrays can be used to identify a disease in an individual. SNP microarrays provide an efficient and inexpensive tool for simultaneously studying multiple genetic variations in multiple samples.

Design of Microarrays

The following sections discuss the design of the three types of microarrays described above and the suitability of the materials used for the microarray probes for each of the three types.

GENE EXPRESSION MICROARRAY

These microarrays are the most common type of microarray in use today. The array elements consist of either cDNA derived from mRNA of known genes but of unknown sequence, or oligonucleotides for which detailed sequence information is available. Oligonucleotides are preferred array elements because of the affordable cost of synthesis and the large amount of sequence information now available for specific genes or gene fragments. These can be arrayed in specific patterns to enable

accurate analysis of related gene sequences and gene families in a single hybridization assay. The following general principles apply to oligonucleotide design for gene expression microarrays:

(1) Oligonucleotides should be 25–70 mers.
(2) Oligonucleotides should include appropriate controls (i.e., oligonucleotides corresponding to sequences from a different organism).
(3) All oligonucleotides should map to within 1000 nucleotides of the 3′ end of cDNAs and should correspond to the coding strand.
(4) Sequence repeats, stretches of polyA, G, C, and T and extremes of T_ms should be avoided.
(5) Oligonucleotides should be compared to sequences in existing databases to avoid cross-reactivity (less than 70% sequence identity with nontarget sequences is preferable).

In addition to oligonucleotides, PCR amplicons and double-stranded DNA (dsDNA) are also used as probes. However, the PCR amplicons require purification to remove enzymes, salts, nucleotides, and other contaminants from the amplification process that could interfere with the binding of the probes and could also inhibit hybridization. In addition, the preparation of dsDNA probes for spotting is labor intensive and expensive. Moreover, dsDNA probes can have repetitive sequences that compromise hybridization specificity. When sequence information is unavailable, dsDNA remain the probes of choice because unknown dsDNA probes can still be used to study gene expression.

aCGH MICROARRAYS

These microarrays traditionally use bacterial artificial chromosomes (BACs) of 100–200 kilo-base pairs per DNA segment as the array elements. However, the large-scale DNA isolations or PCR amplifications of such large-insert clones are elaborate and time consuming. As is the case in expression profiling applications, aCGH microarrays have transitioned from dsDNA targets to oligonucleotide targets. Oligonucleotide libraries or ready-made microarrays can now be purchased, saving considerable time and effort.

SNP MICROARRAYS

Depending on the application, SNP microarrays can use both amplicons and oligonucleotides as probes. In one of the most common formats to detect mutations in a gene sequence, the probe is that of a single gene in which the sequence differs by a single nucleotide polymorphism from the sequence of the other probes for that gene in the same microarray. For the discrimination of only one mismatch, short oligonucleotide probes (15–30 bp) maximize the destabilization caused by mispairing and are therefore used for the detection of SNPs.

Manufacturing of Microarrays

Microarray elements are deposited onto a solid support, the most widely used of which is glass. Microarray manufacturing can be divided into two main categories, direct synthesis of the probes on the microarray (in situ) or synthesis of the probes before spotting on the microarray (ex situ). In situ synthesis is generally used for higher density microarrays but is limited to nucleotides of approximately 25–100 bases. With increasing nucleotide length, the likelihood of truncated products increases because of the limited stability of building oligonucleotides in situ. In contrast, ex situ microarray manufacturing can put any premade material into a microarray format, including oligonucleotides, PCR products (amplicons), complementary DNA (cDNAs), and BACs.

The main techniques for in situ synthesis are photolithography, maskless lithography, and ink jetting. Microarrays are generally manufactured commercially, although for a small number of low-density microarrays, the end user can manufacture the microarrays using a low-throughput microarray manufacturing robotic instrument (a personal microarrayer). However, only maskless lithography and ink jetting are available for end user manufacturing. In photolithography, a glass substrate containing a photomask, which is chemically prepared so that particular nucleotides bind to specific positions, is used to synthesize the oligonucleotides on the substrate. The masks predetermine which of the nucleotides are activated when flooded with one of the four types of nucleotides. The process is repeated until the required number of bases is synthesized. The manufacture of these microarrays uses computer algorithms and multiple spots to cover the gene of interest. Maskless lithography uses a digital micromirror device that uses a solid-state array of miniature aluminum mirrors to create virtual masks that replace the physical photomasks. A computer controls the desired pattern of UV light via individual mirrors. Each digital micromirror in turn controls the pattern of UV light projected onto the glass in the reaction chamber, which is coupled to a DNA synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next nucleotide will be coupled. The patterns are coordinated with the DNA synthesis chemistry in a parallel, combinatorial manner so that hundreds of thousands of unique oligonucleotides can be synthesized in a single microarray. Ink jetting is accomplished by building up the nucleotides, base-by-base, in repetitive print layers using standard phosphoramidite chemistry. Inkjet heads similar to those used in commercial inkjet printers are connected to bottles that contain the four different phosphoramidite nucleotides that make up the building blocks of in situ nucleic acid synthesis. The advantages of inkjetting and maskless lithography are flexibility in design and the ability to make small batches of arrays quickly.

The two main types of ex situ manufacturing techniques are microspotting pins (contact printing) and piezoelectric printing (noncontact). The technology excels at printing multiple probes many times over numerous surfaces with one small-volume loading of probe. Spot size and delivery volume are controlled by the size of the end of the tip, and many tip sizes are available. A piezoelectric printing mechanism uses a small dielectric crystal in contact with a glass capillary that holds the sample fluid. Application of the voltage results in ejection of fluid from the tip, resulting in drop volumes from hundreds of picoliters to several microliters.

General Experimental Considerations

Regardless of the type and application, all microarray experiments have a similar workflow: amplification step, labeling, hybridization, and wash steps, followed by scanning, quantitation, and reporting. The experimental design determines the type of microarray used, number of spots required, and the specific sets of nucleic acids on the microarray. The experimental design also influences the platform used, such as the number of spots, surface type, nucleic acid type, throughput, resolution, and number of colors that can be detected in a single assay. Platforms can be open (support is available from multiple vendors) or closed (support from a single vendor). In general, experimental designs that require a high density of spots and quantitation are more difficult and expensive to implement than qualitative assays.

Microarray Sample Considerations

Sample extraction, isolation, and preparation should be carefully chosen in order not to alter the ability of the resulting target to hybridize to the microarray. In general, sample preparation issues are the same for microarrays as for other laboratory techniques such as qPCR (quantitative PCR) and sequencing (described in chapters ⟨1126⟩ and ⟨1127⟩). RNA, cDNA, genomic DNA, and PCR products are some of the sample types analyzed with microarrays. In some genotyping applications, specific alleles are used both as array elements and targets.

As with any nucleic acid technique, the quality of the nucleic acid is critical for the microarray experiment. The nucleic acid should be pure, intact, and accurately quantitated before use ⟨1126⟩. In particular, the presence of contaminating DNA in total RNA samples may cause problems in microarray analysis because some labeling methods label both RNA and DNA with equal efficiency. For some applications in which even trace contaminants with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. For example, contaminating, labeled DNA can hybridize with microarray targets leading to high-level hybridization signals that are not derived from RNA transcripts, thus resulting in an inaccurate estimation of the target RNA concentration because both nucleic acid species are quantitated at the same wavelength.

A major consideration in any microarray experiment is the availability of adequate amounts of sample nucleic acid for analysis. For example, sample from laser-capture microdissection, needle tissue biopsies, or other small clinical samples do not yield sufficient RNA (for expression microarrays) or DNA (for aCGH microarrays) and must be amplified before analysis. It is critical that the amplification procedures for amplification of mRNA be so designed that the final mixture of amplicons accurately reflect the distribution of mRNA species in the sample. Uniform amplification of genomic DNA for aCGH microarrays can be achieved by the use of multiple displacement amplification (MDA), which overcomes the nonuniform amplification of genomic DNA that occurs in PCR-based amplification methods that use degenerate oligonucleotide primed PCR (DOP-PCR). For SNP arrays where specific alleles are the target of interest, nonuniform amplification is not an issue, and samples can be amplified (and labeled) by PCR, multiplex PCR, and WGA (see ⟨1127⟩).

Microarray Labeling

The targets for a microarray are a population of nucleic acids that are extracted from a sample and are appropriately labeled. Many methods can be used for labeling targets (see ⟨1127⟩), but fluorescent labeling is the most widely used because it offers high sensitivity and a superior dynamic range. An added advantage is the ability to detect two or more signals in a single experiment. The method of labeling depends on the microarray type. The two methods used to fluorescently label targets for gene expression microarrays, direct and indirect labeling, have been described in ⟨1127⟩. In general, the second method (indirect labeling), in which the label is added via a linker, requires less starting material and is less expensive. Published reports have shown that this method yields results similar to those obtained from directly labeled samples. In microarray aCGH, a patient's DNA and reference DNA (300–1000 ng) are typically fluorescently labeled with red and green fluorescent dyes, respectively, often using a random priming protocol. Random prime labeling uses a high concentration of Klenow enzyme whereby genomic DNA is digested with restriction enzymes and hybridized with random primers. The primers are extended by the 5′–3′ polymerase activity of Klenow, resulting in a strand displacement activity with the direct incorporation of labeled nucleotides. SNP microarrays using oligonucleotides as array elements are labeled using fluorescently labeled nucleotides in both single and multiplexed PCR reactions, followed by a purification step to remove unincorporated dyes. Where amplicons are used as array element, labeled oligonucleotide probes are synthesized using phosphoramidite chemistry.

Hybridization and Wash

Hybridization should be carried out under conditions that minimize annealing of noncomplementary fragments. The wash steps following a hybridization reaction are optimized to provide the highest possible specificity, signal-to-noise ratio, and reproducibility (see ⟨1126⟩). Before hybridization, double-stranded probes and targets should be denatured, and nonspecific sites should be blocked. Microarray surface chemistries are designed to capture all nucleic acids with high efficiency, so the free-binding groups on the surface must be blocked or inactivated to prevent nonspecific binding of labeled material that could compromise the signal-to-noise ratio. Surfaces are blocked and washed with various aqueous-based buffers that typically include salts, detergents, and blocking agents such as low molecular weight, hydrolyzed proteins. The purpose of the posthybridization washes is to remove all unattached and nonspecifically bound label from the surface and probes. In general, both automated and manual washes are done in saline sodium citrate/sodium dodecyl sulfate (SSC/SDS) buffers of various concentrations and at different elevated temperatures depending on the stringency required. After the final wash step, microarrays using fluorescent targets are dried immediately by centrifugation or in a nitrogen stream. Hybridized microarrays must be stored in the dark and should be scanned as soon as possible. Some fluorescent dyes used in microarray analysis are subject to degradation by environmental ozone, and in these cases ozone levels in the experimental environment must be less than 5 parts per billion. Specialized ozone-free hoods are made to protect microarray dyes.

Microarray Detection

Regardless of the microarray type, each spot on a microarray represents a unique probe sequence to which a single, labeled target is bound, and this specific binding allows detection and quantitation of the target. This is achieved by the emission of light (photons) at a particular wavelength by the fluorescently labeled duplexes when the microarray is exposed to light of specific wavelength from an excitation source. The emitted fluorescent light is converted to electrical energy by a detector. The detector is either a photomultiplier tube (PMT) or a charge-coupled device (CCD) with specially designed optical paths that collect the raw data from microarrays (scanning). The detector filters and optical paths are designed to detect specific fluorescent dyes at sufficient resolution while eliminating crosstalk when two or more dyes are used on a single microarray. The resulting signal is proportional to the number of photons emitted by the microarray. These signals are used to create a digitized image showing the presence and quantitation of specific targets.

Samples can be scanned from a single wavelength channel or can be sequentially scanned from two channels. For instance, for a single-channel microarray platform, a sample is typically labeled with a fluorophore that emits a signal in the red channel. For a dual-channel microarray format, a second sample can be labeled with a dye that emits in the green channel. Dual labeling is used in some experimental designs, such as expression microarrays, to measure the overexpression of a gene associated with a disease state. In such experiments, cDNAs derived from the mRNA of normal and diseased tissues are differentially labeled, mixed, and tested on the same slide in a competitive hybridization reaction. The resulting ratios of the two colors reflect the relative abundance of the labeled material within each sample. Similarly, calculating the fluorescent ratios from each target on an aCGH microarray allows the mapping of gains and losses for a chromosome of interest.

Microarray Image Processing

Most microarray scanners detect and acquire one, two, or more colors (via one, two, or more channels). The optical path of the system minimizes overlap between the spectra (crosstalk) and allows acquisition of two spectrally separate images. In many cases, the images are represented as a red and a green image. When two colors are used, the ratio of the two fluorescence images eliminates artifacts caused by regional bias and irregular spot size. When one color is used, the fluorescence signals from two or more microarrays are normalized and can be compared with each other. Diameters of spots printed on the arrays range from 10 µm to just under 1000 µm, and the resolution of scanners ranges from 1 to 50 µm. Thus, depending on scanner resolution, variable amounts of pixel data can be collected per scan over an entire microarray.

MICROARRAY IMAGE ANALYSIS

The analysis of scanned images usually involves three tasks: spot finding or gridding, image segmentation, and spot quantification.

Spots are initially assigned specific coordinates, and the process of spot finding or gridding can range from manual to fully automatic, depending on the image-processing software used. This takes into account the individual size and shape of each spot and adjusts for uneven rows and columns that may be produced by the printing process.

The process of segmentation partitions the entire image to foreground or background pixels and relies on the spatial and intensity properties of each pixel. There are four main types of signal segmentation that have been used for spotted arrays. The simplest method is spatial segmentation which places two circles (inner and outer circles) of fixed but different sizes over each spot to demarcate probe signal from the immediate background signal. On the one hand, because of the irregularity of spot sizes on some microarrays, the actual area inner circle may be larger than the diameter of a spot and thus will contain background pixels. On the other hand, artifacts and signal can be found in the area between the inner and outer circles and con-

tribute to the background signal. The second method, intensity-based segmentation, distinguishes signal pixels from background pixels based on the spot intensities within a target region. In this case, a certain percentage of pixels within the top-ranked intensities may be classified as signal pixels. The advantages of this method are simplicity and speed, but the drawback is the inability to distinguish between artifacts and signal and the tendency to detect low signals that are close to background. The third method is a statistical approach known as Mann-Whitney segmentation that combines information from spatial and intensity-based analysis. Here, background pixels located outside the inner circle set are used to determine a threshold intensity level for a signal within the inner circle. The limitation of this method is that a large amount of spot irregularities and artifacts can reduce its accuracy. The fourth method, the trimmed measurement segmentation method, also combines spatial and intensity information and measures signal distributions inside and outside the inner circle. The method trims the upper and lower extremes of each distribution to allow removal of signal from artifacts and incorrectly located background or foreground signal pixels.

The main assumption of spot quantification is that the total fluorescent intensity from a spot is proportional to the expression level of the labeled transcript. This is highly dependent on a number of factors, including target preparation, hybridization conditions, and signal detection within the linear dynamic range. If the amount of probe deposited during the microarray manufacturing procedure varies from spot to spot and from array to array, thus resulting in different sized spots, the sum or total signal intensity can be variable and inaccurate. To correct for this variation, microarrays should be spotted via homogenous surface chemistry that has a fixed binding capacity. This ensures the same amount of probe at each spot location. Alternatively, spots can be quantified by taking the mean, median, or mode of intensities of all signal pixels determined to be foreground signal. The more robust methods that protect against outlier signals are the trimmed mean (where a certain percentage of top and bottom signals are trimmed before calculation of the mean) and median signal intensities. When two different fluorophores are used, the intensity ratio can be used to correct for variable probe amounts and can be calculated from mean, median, and mode intensities from each channel.

MICROARRAY DATA ANALYSIS

Particularly dense formats of microarrays that contain tens of thousands to millions of probes per chip or slide generate a large volume of raw data per array, which requires the use of specialized data-analysis software. Microarray software programs are designed to extract primary data, normalize the data to remove the influence of experimental variation, and link probes to relevant gene and sequence-derived targets. Software programs are also available to apply statistical methods, analyze, visually display, and manage data in order to extract biologically meaningful information. The major parts of data analysis are normalization, background correction, and ratio calculation.

Normalization systematically adjusts microarray raw data in an effort to reduce the variability brought about by differences in the manufacture and processing of the microarrays and by technical variables so that true biological differences between samples can be detected. The wide range of normalization methods precludes a detailed discussion of the topic in this chapter, and currently there are no standards for normalization. Commonly used algorithms are selected based on the microarray type, the number of fluorophores used, and the samples being studied. Some methods are built into the manufacturer's software, but others are available from commercial sources or open-source software providers.

Background correction eliminates low levels of noise in microarrays stemming from both the inherent noise of the detection instruments and from the surface chemistry used in manufacturing. Several contaminants acquired from microarray processing can cause high levels of background that must be corrected before data analysis.

In two-color microarrays, the ratio of signal intensities of array elements of two co-hybridized samples is used as a relative measure of gene expression. In single-channel systems, the ratio can be calculated between signals taken from two different samples (one sample is a reference sample) hybridized on individual microarrays. Thus, the resulting data from microarrays does not represent an absolute quantification but rather a relative level of RNA or DNA against a reference sample or control.

Quality Control and Quality Assurance

As with any diagnostic assay, quality control and quality assurance are critically important. Microarrays must demonstrate robustness and reproducibility. The general quality control and assurance steps outlined in chapter ⟨1127⟩ for nucleic acids and NAT also apply to microarrays. Unlike other diagnostic tests, no reference reagents are available at present for quality control of microarrays, and regulatory guidance is emerging. FDA has issued a draft guideline titled "In Vitro Diagnostic Multivariate Index Assays." This guidance addresses the definition and regulatory status of a class of in vitro diagnostic devices referred to as in vitro diagnostic multivariate index assays (IVDMIAs), and microarrays fall into this category. The guidance addresses premarket pathways and postmarket requirements with respect to IVDMIAs.

Several unintended sources of variability that are specific to microarrays can extensively affect signal intensities and the accurate derivation of a true signal that accurately reflects the labeled transcript. A major source of variability is spot quality. Measurements of spot quality at the processing stage permit removal of spots with poor or questionable quality. Other sources of variability are artifacts, for example, regional shifts (rise or fall) in an array's overall signal that can be visualized within single chips or in-composite data derived from multiple chips. These changes can be distinguished from actual variability because they are nonrandom, and patterns can be detected by visualizing signals over the entire area of the chip. When dyes of different spectral properties are used to label two different samples in a competitive hybridization reaction using a single array, dif-

ferences may arise because of labeling bias rather than gene expression level. For instance, the green channel may appear consistently brighter than the red channel despite the fact that there are no real differences in expression. Hybridization with reverse dyes can ensure detection and elimination of dye bias effects. As with any quantitative assay for RNA, the integrity of the sample affects its measurement, and sample quality is an important determinant for accuracy. For instance, because labeling is directed from the 3′ to the 5′ end but RNA degrades from the 5′ end, degraded RNA leads to high 3′/5′ ratios, resulting in nonuniform labeling across the entire transcript. Finally, variability can be introduced during the processing of microarrays, which is a relatively complex procedure that involves multiple steps such as labeling, hybridization, washing, and staining (technical variables). Such variability can mask true differences in the samples tested.

When used as a diagnostic test, the microarray should demonstrate robustness, reproducibility, a high degree of correlation to the original format, and reliable prognosis prediction. The microarray ideally should contain at least 2–3 replicate spots for each reporter gene to ensure intra-assay reproducibility. With a two-channel microarray, a reference sample pool can be hybridized in the complimentary fluorescent channel so that data can be expressed as log ratios, which reduces the need for extensive normalization. Interassay reproducibility of test results and stability over time can be tracked by using a number of reference samples that, when labeled and hybridized, represent a spectrum of predictive endpoints (for instance, high risk, borderline risk, low risk) and that should fall within a predetermined range of results. Failure of these controls should result in rejection of results of samples in the same assay run. If the assay is performed at many sites, site-to-site reproducibility is imperative and must be assessed. The reproducibility of the assay with regard to tissue extraction also must be determined, and the quality of tissue specimens or RNA should be specified clearly (for instance, percentage of tumor cells within a specimen).

In conclusion, microarray experiments should be carefully designed and conducted in order to minimize variability and to yield data that accurately relate to the samples analyzed. In addition, biomarkers of interest should be analyzed and verified using an alternative platform such as qRT-PCR that should be shown to be reproducibly detected in the same and different samples. The development of reference standards, especially when microarrays are used as diagnostic tests, is the next step to ensuring the quality and validation of microarray results. With the shift from custom-built to commercial microarrays, issues with reproducibility, standardization, and quality control have been largely addressed by the stringent quality controls used in commercial manufacturing.

⟨1129⟩ NUCLEIC ACID-BASED TECHNIQUES—GENOTYPING

INTRODUCTION

This chapter outlines techniques for detecting single-base DNA differences and other types of polymorphic DNA sequences that occur in the three billion bases that make up the human genome. The most common genetic variation is a single nucleotide polymorphism (SNP), which is a simple change in one base of the gene sequence. SNPs occur on average every 1000 bases and account for a significant amount of inter-individual variability. SNPs can predispose individuals to disease or influence their response to a drug. Approximately 1.8 million human SNP loci have been identified, and more are likely to be discovered in the coming years.[1]

Common approaches for detecting SNPs and other types of polymorphic DNA sequences are described in the following sections. These approaches encompass a variety of techniques, such as nucleic acid amplification techniques (NAT), real-time NAT, and microarrays, the principles of which are covered in more detail in related chapters. This chapter focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences.

SNP GENOTYPING TECHNOLOGIES

Although the usefulness of studying SNPs for gene mapping and disease association studies is apparent, a single standardized procedure for SNP genotyping has not been adopted. Various approaches for performing SNP genotyping have been developed to meet a wide range of needs, including throughput capacity, ease of assay design, accuracy, and reliability. Available procedures can also be divided according to whether they are based on identifying known SNPs or whether they can be used to screen for unknown SNPs. To identify the most appropriate SNP genotyping procedure for a specific application, the throughput requirements in terms of the number of SNPs to be analyzed per sample (multiplexing level) and the sample throughput need to be determined because different approaches may work best depending on these requirements.

Most procedures used for genotyping SNPs depend on polymerase chain reaction (PCR) amplification of the genomic regions that span the SNPs followed by the actual genotyping reaction. PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions limits the throughput of many of the current SNP genotyping assays. The following sections outline several of the major approaches currently in use for SNP genotyping. In many cases the underlying technology

[1] Database of Single Nucleotide Polymorphism (db SNP) Build 128 is available from National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/projects/SNP/index.html.

can be modified to meet the specific application requirements in terms of sample throughput and number of SNPs detected. In general, real-time PCR-based procedures are better suited to higher sample numbers, and array-based procedures are better suited to the simultaneous detection of many SNPs. Newer technologies based on multiplexed array formats are also emerging and will be suitable for high sample numbers and many SNP applications.

Sequencing

Sequencing is the definitive procedure for DNA analysis, and its use for SNP detection allows unambiguous identification of base changes (see *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩ for nucleic acid sequencing). The standard technology is expensive, and the procedure is time consuming and labor intensive and suffers from low sample throughput. Sequencing is a useful confirmatory tool, and it has applications in situations when other technologies are not appropriate, but is not the most cost-effective solution for the majority of SNP genotyping applications that require the identification of only one or a few bases.

Restriction Fragment Length Polymorphism Analysis

The first widely used procedure for the detection of polymorphisms exploited alterations in restriction enzyme sites caused by SNPs, leading to the gain or loss of cutting events. PCR–restriction fragment length polymorphism (RFLP) analysis comprises PCR amplification of a fragment of interest and subsequent digestion with a restriction enzyme. The fragments produced are typically analyzed by a size fractionation procedure, usually gel electrophoresis. Because of its simplicity, the procedure has been and still is extensively used, although it entails certain limitations: only a subset of polymorphisms that reside in an endonuclease restriction site can be studied with the conventional procedure; incomplete digestion due to suboptimal processing can produce misleading digestion patterns; and the procedure is less amenable to automation than are other SNP genotyping procedures.

Probe Hybridization

The basis of many SNP genotyping procedures are DNA hybridizations that make use of the stronger binding of a DNA probe to a perfectly matched complementary target than to a target that contains a single base mismatch. The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in the late 1970s and subsequently was used to detect the sickle-cell mutation in the beta-globin gene by Southern blot hybridization. The invention of PCR facilitated the further development of probe-based assays for genotyping SNPs in complex genomes.

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions but also by the secondary structure of the target sequence and the nucleotide sequence flanking the SNP. Therefore, prediction a priori of the reaction conditions or the sequence of the ASO probe that will allow optimal discrimination between two alleles using ASO hybridization is difficult. These parameters should be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an extremely difficult task.

One approach to counter the problem of assay design is to carry out multiplex ASO hybridization reactions on arrays that carry multiple probes for each SNP that will be analyzed. This involves using probe sets in which the SNP occurs at different positions along the probes. It becomes feasible to include large numbers of ASO probes per SNP when one uses high-density arrays that can carry as many as 10^6 probes per cm^2.

Another approach is to use base analogues such as locked nucleic acid (LNA), which is described in detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. For applications that involve few SNPs but many samples, homogeneous real-time PCR approaches have been developed. These include the use of fluorescent probe chemistries such as hydrolysis probes, stem-loop probes, and FRET (fluorescence resonance energy transfer) hybridization probes. The principle of these assays is discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. For SNP detection, the basis of many assays is the selective binding of the ASO probe to its perfectly matched target sequence, resulting in energy transfer and generation of a fluorescence signal. Probes designed with specific secondary structures tend to form a stem–loop structure that destabilizes mismatched hybrids, increasing their power of allele distinction as compared with that of linear ASO probes. Hydrolysis probes modified with minor groove-binder molecules that increase target affinity show improved powers of allele discrimination. The use of two probes, each labeled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. Limited multiplexing can be achieved by using probes labeled with different fluorophores. In the fluorescent probe–based assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtiter plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR. In this case the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence, and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results.

A third approach involves heating the reaction after PCR has been completed in order to disassociate the probe from the target. Each duplex has its own specific T_m, which is defined as the temperature at which 50% of the DNA becomes single

stranded. The T_m depends on the stability of the probe–target duplex. Perfectly matched probe–target duplexes have a greater stability and hence a higher T_m than does the same duplex containing a single base mismatch. By continuously monitoring the fluorescence during the heating phase, analysts generate a "melt curve" that measures the changes in fluorescence that result when the probe denatures, or "melts," away from the amplicon. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

Because no post-PCR processing or label-separation steps are required, homogeneous real-time PCR assays are simple to perform, making them useful for high-throughput genotyping applications. The optimal probes must be designed individually for each SNP, and the assays are therefore most efficient when a limited number of SNPs is analyzed. The cost of probes modified with fluorescent and quenching moieties may also be a limiting factor in the high-throughput application of the assays.

Primer Extension

In this technique, an oligonucleotide is used to prime DNA synthesis by a polymerase enzyme, as performed in a standard PCR or sequencing reaction. Variations of the technique exist. Allele-specific PCR uses two primers, each fully complementary to one of the SNP alleles, with the SNP position being at the 3′ end of the primer, and with a common reverse PCR primer to selectively amplify the SNP alleles. Because only perfectly matched oligonucleotides will prime DNA polymerase extension, product will be detected only from the reaction containing the perfectly matched primer.

Agarose gel electrophoresis is used to detect the amplified products, although homogeneous, real-time, allele-specific PCR approaches have also been developed using primers labeled with different fluorophores or a fluorescent dye that intercalates with the double-stranded PCR products or by performing amplicon detection using probes such as hydrolysis and hairpin (stem–loop) probes. When using intercalating dyes or labeled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, one may find that the specificity of the procedure may be compromised owing to primer–dimers and other spurious amplification products that will not be distinguished from the actual PCR product. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the hydrolysis and hairpin probe assays, the homogeneous allele-specific PCR procedures are best suited for the analysis of a limited number of SNPs in large sample collections. Array-based approaches for greater SNP multiplexing have also been developed.

In procedures based on single nucleotide primer extension (sometimes known as minisequencing), allele discrimination is based on the high accuracy of nucleotide incorporation by DNA polymerase. A primer is used, and its 3′ end is positioned on the base just preceding the SNP to be tested. The DNA polymerase is then used to incorporate labeled ddNTPs, each labeled with different fluorescent dyes. After the labeled oligonucleotides are separated from the nonincorporated ddNTPs, the results can be scored on a fluorescence plate reader. In addition to fluorescent tags, ddNTPs may be labeled with biotin or haptens and then detected indirectly through antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminiscent markers in ELISA formats.

Multiplexing of this procedure has also been described to reduce costs and improve throughput. In these procedures, the different loci genotyped simultaneously are separated either by gel electrophoresis or by hybridization to arrayed tags. Primer extension directly on a solid support such as a microarray is also possible. The immobilization of the single-stranded primers on the solid support may be through biotin–avidin–streptavidin reaction or covalently via 5′ disulfide groups.

Mass spectrometry using techniques such as matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used to determine the identity of the ddNTP incorporated based on mass. A difficulty with MALDI-TOF MS is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. Such enzyme-assisted procedures have proven to be more robust and to provide more specific allele discrimination than does ASO hybridization at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization is minimized.

Ligation

In the oligonucleotide ligation assay (OLA), oligonucleotides are designed so that they meet at the position of the SNP to be tested. Enzymatic joining, using a DNA ligase, occurs only when the match is perfect. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of the SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microplate wells by colorimetric approaches. Multiplexing and the use of gel separation have also been described.

OLA has also been used in microarray formats with one of the ligation probes immobilized or with immobilized single stem–loop probes. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays or on microparticles that carry a generic set of oligonucleotides that are complementary to a "tag" sequence on one of the ligation probes. In practice, thermostable ligases are frequently used for genotyping SNPs in combination with PCR before allele-specific ligase detection reactions. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET. Compared with DNA-polymerase-assisted primer extension procedures, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides, which increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA). RCA can be used to amplify the ligated circularized padlock probes to a level required for detecting single-copy sequences. A homogeneous, isothermal assay for genotyping individual SNPs in a microtiter plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labeled hairpin primers.

Displacement

The invader assay uses the property of flap endonucleases (FENs) for removing redundant portions (flap) from the 5′ end of a downstream DNA fragment overlapping an upstream (invader) DNA fragment. An invader oligonucleotide is designed with its 3′ end on the SNP to be tested. Two oligonucleotide signal probes are also designed, overlapping the polymorphic site and each corresponding to one of the alleles. After displacement of the signal probes by the invader probe, FEN-mediated cleavage occurs only for the perfectly matched allele-specific signal probe. Generation of the cleaved fragment is monitored by using it in a second reaction as an invader probe to cleave a FRET probe. This assay does not require PCR amplification of the locus to be tested, and scoring can be done using a simple fluorescence plate reader.

Pyrosequencing

In the pyrosequencing procedure, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate (PPi), which is released on incorporation of deoxynucleotide triphosphates. The genotype of an SNP is deduced by sequential addition and degradation of the four nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtiter plate format. Using pyrosequencing, the apparatus can determine short 30 to 50 bp sequences of DNA that flank an SNP. A limitation of the procedure is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes. An advantage of the procedure is that any new polymorphism will be detected. However, specific equipment is needed for the injection of the nucleotides.

Single-Strand Conformation Polymorphism and Heteroduplex Analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis were among the first procedures established for the detection of SNPs. Conventional SSCP analysis involves denaturing PCR-amplified fragments and subsequent formation of sequence-specific secondary and tertiary structures of the single strands during nondenaturing gel electrophoresis. The electrophoretic mobility then depends on the 3-D shape of the single-stranded molecules. One single base difference in DNA fragments of up to 300 bp will usually change the conformation in a way that can be detected by nondenaturing PAGE.

The traditional polyacrylamide gels and ^{32}P-labeled fragments are frequently being replaced by fluorescently labeled fragments and automated capillary electrophoresis. The simplicity of the procedure, combined with automation and short analysis time, contribute to high-throughput analysis at relatively low cost. If the denatured PCR products are allowed to slowly renature, they form DNA duplexes. The duplexes with the same sequence on both strands (homoduplexes) or with a single base pair mismatch on one strand (heteroduplexes) have different electrophoretic mobility in a native gel. In the case of a single base pair substitution, the heteroduplex can easily be separated from a homoduplex.

In other versions of the technique, denaturing high-performance liquid chromatography (DHPLC) is used for the separation of the heteroduplex and homoduplex strands. The mutation analysis with DHPLC can be almost totally automated with an autosampler on one end and a fraction collector on the other. Analysis is rapid (about 5 minutes per sample), and simple evaluation of data distinguishes between simple and multiple peaks in the elution profiles, allowing lengths as large as 1.5 kb of DNA to be analyzed. A disadvantage may be the recommended use of Pfu DNA polymerase, which, as a high-fidelity enzyme, allows sharper peaks but may be less successful in amplifying some regions.

Short Tandem Repeat Profiling

A short tandem repeat (STR) is a type of DNA polymorphism that occurs when a pattern of two or more nucleotides is repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 bp (e.g., $CATG_n$ in a genomic region) and is typically in the noncoding intronic, or upstream/downstream regions. By examining several STR loci and counting how many repeats of a specific STR sequence there are at a given locus, one can create a unique genetic profile of an individual. Currently more than 10,000 STR sequences in the human genome have been published. STR analysis has become the prevalent analysis procedure for determining genetic profiles in forensic cases. STR analysis in the field of forensics came into popularity in the mid to late 1990s. The STRs in use for forensic analysis are tetra- or penta-nucleotide repeats (4 or 5 repeat units) because these give a high degree of error-free data while being robust enough to survive degradation in nonideal conditions. Shorter repeat sequences tend to suffer from artifacts such as stutter and preferential amplification; sever-

al genetic diseases are associated with tri-nucleotide repeats, including Huntington's disease. Longer repeat sequences suffer more highly from environmental degradation and do not amplify by PCR as well as do shorter sequences.

The analysis is performed by extracting nuclear DNA from the cells of a forensic sample of interest and then PCR amplifying specific polymorphic regions of the extracted sample. Once these sequences have been amplified, they are resolved either by gel electrophoresis or capillary electrophoresis, which allow the analyst to enumerate the repeats of the STR sequence in question. If the DNA is resolved by gel electrophoresis, the DNA can be visualized either by silver staining or an intercalating dye such as ethidium bromide or, as in most modern forensics labs, by fluorescent dyes. Instruments built to resolve STR fragments by capillary electrophoresis also use fluorescent dyes. In the United States, 13 core STR loci have been selected as the basis by which an individual genetic profile can be generated. These profiles are stored in local, state, and national DNA databanks such as the Combined DNA Index System (CODIS).

Forensic reference materials are available. The DNA Profiling Standard is composed of well-characterized human DNA in two forms: genomic DNA and DNA to be extracted from cells spotted onto filter paper.

ASSAY VALIDATION CONSIDERATIONS

The difficulty in reproducing and validating existing and emerging SNP genotyping assays due to factors such as variation in performance of PCR thermal cyclers, efficiency of different enzymes, personnel, and the presence of PCR inhibitors in the sample matrix (discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩ for general NAT assays) can hamper appropriate implementation of the technologies. Also, in the clinical laboratory the use of in-house assay formats often makes comparisons between laboratories difficult. Incorrect diagnosis of a genetic mutation can have significant consequences, so accuracy of 99.99% or higher is essential for such assays. To determine the accuracy of a technology, the new procedure should be validated on multiple samples in which the genotype has been previously determined with a gold standard procedure, such as sequencing. Even with the most accurate procedure of analysis, sample preparation and amplification and detection procedures must be optimized to eliminate any potential inaccuracies.

Some genotyping errors can be minimized by careful planning of the laboratory procedures, the inclusion of well-defined controls, and increased automation. However, errors due to the processes used for genotyping are sometimes difficult to overcome and need to be taken into account. The types of errors and the frequency with which they occur differ between different approaches. Situations in which preferential amplification of one allele or nonspecific probe hybridization occur can all result in SNP miscalls. Additional unanticipated polymorphisms present within the primer/probe sequences can lead to amplification bias, highlighting the need for careful assay design and validation using alternative techniques. Limited and degraded samples can also result in preferential allelic amplification due to chance PCR priming events at low copy number.

It is preferable to have a no-call result, which would require the test to be repeated, than a miscall that provides incorrect results that are subsequently reported. Performance of replicate assays may also help to ensure accuracy. Data interpretation can also affect accuracy. Wild-type, heterozygous, and homozygous mutant results should be clearly distinguished from one another, and a well-defined measure of uncertainty should be attributed to them. Proficiency testing schemes and ring-trials go some way toward ensuring that individual assays are fit for the purpose for which they are intended for specific applications and that the staff performing them are competent. Sharing of technical information for assay design and sample preparation will also help. The availability of reference panels of well-characterized samples aids assay design and evaluation and allows sound interlaboratory comparisons to be made.

⟨1130⟩ NUCLEIC ACID-BASED TECHNIQUES—APPROACHES FOR DETECTING TRACE NUCLEIC ACIDS (RESIDUAL DNA TESTING)

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. This chapter covers the analytical procedures used to quantify residual DNA in biopharmaceuticals.

Process characterization and the theoretical safety concerns associated with process-related impurities highlight the need for residual DNA testing in biopharmaceutical products. The ability of a manufacturing process to remove residual DNA from a biopharmaceutical product is an indicator of the quality and consistency of the process and that the process is under control. Additionally, the cells used to produce a biopharmaceutical can be sources of a range of complex, heterogeneous, and potentially unsafe impurities, with host cell DNA among these impurities. For continuous cell lines, the potential risk of residual DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence of an infectious viral genome in the cellular DNA of the cell substrate. The oncogenicity activity of residual DNA could arise through its capacity to induce a normal cell to become transformed, which may lead to tumorigenicity. Although animal testing has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans. Residu-

al DNA content, up to 10 ng of residual DNA per parenteral dose, may be considered for DNA originating from mammalian cell cultures, but the acceptable residual DNA content may vary depending on the source of the residual DNA and the route of administration of the product. One can address residual DNA in biopharmaceutical processes in two ways: 1) by validating clearance during process validation; and/or 2) by monitoring residual DNA levels through routine testing of the drug substance. Generally, 10 ng per dose is the accepted limit, by health authorities, of host residual DNA derived from mammalian cell cultures. The level of concern regarding residual DNA can be tied to its source and the route of administration, so the residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation with regulatory agencies. Regardless of whether routine testing of a drug substance is used to determine residual DNA content or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA are required. DNA amplification techniques, such as quantitative PCR (qPCR), are used most often for residual DNA testing because of their superior sensitivity and unique advantages (e.g., high specificity). The expectation is that the analytical procedure used to quantify residual DNA in biopharmaceuticals has a detection limit well below the DNA level allowed by regulators for biopharmaceuticals (often 10 ng/dose). Assays based on hybridization, DNA-binding protein, and qPCR are typically the techniques of choice because they can meet the sensitivity expectation.

SAMPLE PRETREATMENT

Analysis of residual DNA requires accurate quantification of picogram levels of DNA in mg (or larger) quantities of product, which may be in a variety of matrices. In certain circumstances, the sample can be analyzed neat in the analytical procedure with acceptable recovery and precision. When the product or other sample components interfere with the assay sample, dilution may be all that is required to overcome the interference, so long as the specified DNA content of the sample remains within the useful range of the analytical procedure. When sample dilution is not effective in reducing assay interference, it may be necessary to use more extensive sample pretreatment procedures, such as proteolytic digestion, chemical dissociation, or extractions. It may be necessary to use a combination of different pretreatment steps to remove interference to an acceptable level. Extensive sample manipulation can lead to losses of DNA or introduction of environmental DNA, and should be a consideration when using one or more sample pretreatment steps. Contamination with environmental DNA may only be a concern when using a residual DNA procedure that is not sequence specific.

Protein samples may only require digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to quantitatively recover the residual DNA. It may also be possible that the DNA is bound to the sample components, and chemical dissociation (e.g., detergents) may disrupt the binding, allowing sufficient recovery in the residual DNA assay. Residual DNA test procedures often use protein reagents and the use of a chemical dissociation reagent. These materials must be used at a sufficiently low level or removed so that the analysis is not compromised.

It may be necessary to extract the DNA from the sample to remove the inhibitory substances that are causing the reduced DNA recovery. Extraction procedures are typically based on precipitating the DNA from the sample or DNA-specific binding to a matrix (e.g., magnetic beads). Historically, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples before analysis, but the phenol/chloroform extraction technique might not be the best choice for the low levels of DNA typically found in biopharmaceutical samples. Because of these low levels, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if the phenol/chloroform extraction technique is used. Commercial kits are available and have been used successfully for pretreatment of residual DNA samples for improved recovery in the residual DNA assay. For example, some kits use a chaotrope (sodium iodide) and a detergent (sodium *N*-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol. Extraction of DNA from the sample, based on binding to a solid matrix, can be found in various formats. One of the most popular formats uses magnetic beads, where the beads are added to a sample with a binding solution to capture the sample DNA on the beads. The beads are then captured and held in the sample tube using a magnetic stand while the supernatant containing the interfering substances is removed and discarded. The beads are washed repeatedly using a magnetic stand and a wash solution. Finally, the DNA is eluted from the beads for the assay using an elution buffer, with the beads being removed from the sample preparation using the magnetic stand.

The sample manipulation involved with pretreatment may reduce the recovery of the residual DNA or introduce environmental DNA into the sample. Great care must be taken during any sample manipulations to avoid DNA losses or contamination. The addition of target DNA-spiked samples in the residual DNA assay is a common practice. The target DNA-spiked sample should not be confused with the internal positive control (IPC), which is typically a nontarget DNA added after the sample pretreatment step to detect the presence of PCR inhibitors and to evaluate DNA amplification during the analysis. The IPC may also be introduced before the extraction to improve the control of this step. A recovery of 50%–150% of the spiked target DNA is often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects or sample preparation method) make achieving a recovery acceptance criterion of 50%–150% impractical, then correcting the observed DNA concentration by using the load recovery percentage is also an acceptable approach.

HYBRIDIZATION-BASED RESIDUAL DNA ASSAY

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured, labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against autoradiography film for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. The level of hybridization can be measured using a phosphor-imaging system. If the probe has a fluorescent label, the intensity of the spots is determined using a fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semiquantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure.

1. The first step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease.
2. During the second step, the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane.
3. During the third step, the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample.
4. In the fourth step, the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

POLYMERASE CHAIN REACTION TECHNIQUES

Real-time qPCR is a procedure that is well adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe and primers used in the analysis are key to the procedure. The most common qPCR method for detection of this amplification is referred to as the 5′ nuclease assay. In this format, the probe has a reporter dye attached to one end and a quencher dye attached to the other end. A pair of DNA primers is also added to the reaction. During the amplification reaction, a thermostable DNA polymerase initiates DNA synthesis where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA, synthesizing new complementary DNA. While following the template DNA, DNA polymerase I cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe, DNA polymerase I will cleave the probe. The reporter dye is released into the solution and, in the absence of the quencher dye, the resulting fluorescence is measured. Repeating the reaction cycle results in an amplification of the fluorescence signal. The number of cycles required for the fluorescence measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing the fluorescence obtained from a sample to a standard curve, analysts can quantify the residual DNA in the sample.

Alternative Detection Strategies

A number of innovative detection strategies have been developed and commercialized beyond that described above. A few of the most common are as follows:

1. Intercalating cyanine dyes fluoresce after binding to double-stranded DNA. The amount of dye incorporated is proportional to the amount of target amplicon generated. These dyes are inexpensive and easy to use. The disadvantage of this technique is the lack of a specific probe to confer sequence specificity beyond that afforded by the primers, and the dye will also bind somewhat to single-stranded DNA and RNA molecules. Consequently, primer dimers or nonspecific prod-

ucts may affect the quantification. However, it is possible to check for the specificity of the system by running a melting curve at the end of the PCR run, based on the principle that every product has a different dissociation temperature and depending on the size and base content.

2. Other sensitive probes exist that contain a stem-loop structure with a fluorophore and a quencher at their 5′ and 3′ ends, respectively. The stem is usually six bases long, mainly consisting of cytosines and guanines, and holds the probe in the hairpin configuration. The "stem" sequence keeps the fluorophore and the quencher in close proximity, but only in the absence of a sequence complementary to the "loop" sequence. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target, leading to separation between the fluorophore and the quencher, and the probe fluoresces. The amount of signal is proportional to the amount of target amplicon sequence. The increase in fluorescence that occurs is reversible, because there is no cleavage of the probe. It is also possible to design the stem structure to add specificity to this type of probe. However, these probes are often expensive, and the signal can be weak due to the limited possible physical separation between the fluorophore and the quencher.
3. A variation on the second example described above uses a single-stranded nucleic acid sequence containing the specific PCR primers, the specific probe with a stem-loop tail separating a fluorophore and a quencher, and a blocking group. The stem-loop tail is separated from the PCR primer sequence by a "PCR blocker", a chemical modification that prevents the polymerase from copying the stem-loop sequence of the primer. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched, and an increase in signal is observed. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in close proximity. Enzymatic cleavage is not required, nor a separate probe hybridization step, thereby reducing the time needed for signaling. This can result in stronger signals and lower background with faster cycling; however, these probes can be quite expensive and complicated to design.
4. Fluorogenic minor groove binder probes are short linear probes that have a minor groove binder with a nonfluorescent quencher on the 5′ end and a fluorophore on the 3′ end. The minor groove binder prevents the exonuclease activity of the DNA polymerase from cleaving the probe. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close together. The probe is elongated when bound to its target and quenching is decreased, leading to an increase in fluorescent signal proportional to the amount of accumulated amplicon. These probes are also expensive and can produce a low signal-to-noise ratio.

Quantitative Multiplex PCR-Based Residual DNA Assay

An evolution of qPCR is quantitative multiplex PCR, where several pairs of primers and the corresponding probes are introduced in the reaction medium to simultaneously detect multiple targets. Benefits include higher throughput and better control of false-negative results, whereas disadvantages come from amplification and detection interferences, as outlined in *Nucleic Acid-Based Techniques—Amplification* 〈1127〉. One of the applications of this technique is a duplex qPCR, where the introduction of an exogenous DNA, called IPC (internal positive control, see *Sample Pretreatment* above), enhances confidence in the accuracy of the analysis when appropriately amplified. Multiplex qPCR is not used as often as single-target qPCR to assay host cell residual DNA in biopharmaceuticals.

RESIDUAL DNA TESTING POINTS TO CONSIDER

When developing a residual DNA assay, one should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. In addition, environmental, health, and safety aspects should be considered. Traditionally, hybridization assays were performed using phosphorus (^{32}P)-labeled DNA and autoradiography. Because ^{32}P decays quickly, probes prepared with ^{32}P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome. These issues with ^{32}P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this process represents a semiquantitative assay, but if the intensity of the spots is determined using a densitometer or other imaging system, the results may be quantitative. DNA-binding protein assays and qPCR give quantitative results. Quantitative assays are typically preferred instead of semiquantitative assays (e.g., older hybridization-based methods), because the results are considered more accurate and precise, which allows better process monitoring and control. Because of sample matrix interference, a sample pretreatment step is often required to obtain accurate and reproducible results. Pretreatment steps can influence the recovery of DNA, so it is usually necessary to design the assay with a spike-recovery control with an acceptance criterion to ensure assay performance.

In-house controls are usually prepared in the laboratory and qualified by ultraviolet spectroscopy, using standard techniques employed in molecular biology, to determine the DNA content and purity. The hybridization assay uses genomic and/or vector DNA, labeled randomly throughout the DNA, as the hybridization probe reagent. For this reason, the hybridization assay is specific for the source of DNA but is not specific for a given sequence. A synthesized probe, specific for a specific sequence, can be prepared and used in the hybridization assay if this level of specificity is desirable. The DNA-binding protein residual DNA assay is not sequence specific and hence not specific for the host DNA. Therefore, laboratory personnel should avoid contaminating samples for this assay with environmental DNA before denaturing the DNA; otherwise, the DNA result may be

falsely elevated. The qPCR probe has the advantage of being sequence specific, but this creates some special challenges for development of a qPCR residual DNA assay. The qPCR-specific sequence must be a stable sequence within a suitable region of DNA. The recovery of the target sequence must consistently represent the recovery of all the residual DNA. Biopharmaceutical manufacturing processes may typically include operations that shear DNA into smaller fragments, and this must be taken into consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. When bridging from one DNA assay technique to another, a thorough understanding of the DNA analyte is critical. Some assays can detect both single-stranded and double-stranded DNA, whereas some can only detect double-stranded DNA (e.g., some fluorescent dye-binding assays). There are assays that are not sequence specific, and those assays that are sequence specific can be influenced by the copy number of the target sequence present in the DNA. There are assays that require two or more antibody molecules to bind to the DNA fragment (e.g., DNA-binding protein-based residual DNA assay), and if the DNA fragments are too small and present in sufficient quantity, they can saturate the reagents and inhibit the assay (hook effect).

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and provides valuable characterization of the manufacturing process.

⟨1132⟩ Residual Host Cell Protein Measurement in Biopharmaceuticals

1. INTRODUCTION AND SCOPE

Many medicinal products are produced through recombinant technology via a host cell (e.g., bacteria, yeast, or mammalian, insect, or plant cell lines). During the manufacture of such products, some amount of non-product, host cell-derived material will inevitably be introduced into the process stream. This process results in a mixture of the desired product and host cell-derived impurities, including host cell proteins (HCPs), and other process-related impurities that will be targeted for clearance through bioprocessing.

Residual HCPs have the potential to affect product quality, safety, and efficacy; therefore, the quantity of HCPs should be low. The product purification processes must be optimized to consistently remove as many HCPs as feasible, with the goal of making the product as pure as possible.

The primary concern with HCPs in biopharmaceutical products is their potential to induce anti-HCP antibodies that could induce a clinical effect in patients. In addition, HCPs may possibly act as adjuvants, which can induce anti-drug antibodies that can affect the safety or efficacy of the drug. A more extensive discussion of immunogenicity and its effect on preclinical and clinical studies is described in *USP* general chapter *Immunogenicity Assays—Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* ⟨1106⟩. HCPs can also have a direct effect on the quality of the product itself. For example, proteolytic HCPs, even in minute quantities, can cleave the desired protein product over time, reducing or eliminating biological potency or altering stability.

This chapter focuses on HCP immunoassays for recombinant therapeutic products. It does not address products such as vaccines or gene-, cell-, or tissue-based therapies, although the general principles discussed may apply to the measurement of HCPs in these products. The design and validation of immunoassays for HCPs involve unique and significant challenges due to: 1) the wide variety of possible HCPs in medicinal products; 2) the general use of polyclonal antibody reagents to detect them; 3) the lack of exactly matched standards for quantitation; 4) in some cases, a considerable effect from sample dilution effects; and 5) inherent limitations to measure single HCP species.

The chapter includes assay development strategies throughout the product and process development lifecycle, and it describes approaches to demonstrate that the assay is fit for use (e.g., illustrates unit operation clearance of HCPs, lot release). Because of the complexity of HCP immunoassays, careful development and characterization of critical reagents are required, particularly for the immunogen used to elicit the anti-HCP antibodies, the antibody reagent(s), and the assay HCP standard. Because HCP testing is an essential part of process development and product quality control, HCP testing is also discussed in conjunction with regulatory requirements and other considerations for guidance on an overall control strategy for HCPs. A brief outline of the general chapter follows:

1. Introduction and Scope
- *1.1 Considerations for Manufacturing, Characterization, and Consistency*

2. Terminology

3. HCP Immunoassay Methods
- *3.1 The Assay Development Cycle*
- *3.2 Development and Characterization of HCP Reagents*
- *3.3 Immunoassay Method Development and Qualifying as Fit for Use*

4. HCP Immunoassay Method Validation
- *4.1 Accuracy*
- *4.2 Sensitivity and Assay Range*
- *4.3 Sample Linearity*

General Chapters

4.4 Specificity
5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement
5.1 Considerations for Electrophoretic Methods
5.2 Considerations for Western Blot Methods
5.3 Considerations for Chromatographic and Proteomic Methods
5.4 Concluding Remarks on Supporting Technologies for HCPs
6. Use of HCP Immunoassays for Process Development, Characterization, and Validation
6.1 Assays for Individual HCPs
6.2. Control Strategy
7. Summary and Conclusions
8. Bibliography

1.1 Considerations for Manufacturing, Characterization, and Consistency

Different cell-based expression systems are used to manufacture medicinal products, such as bacteria (*Escherichia coli, Pseudomonas fluorescens*), yeast (*Saccharomyces cerevisae, Pichia pastoris*), mammalian cells (e.g., Chinese hamster ovary (CHO), mouse myeloma cell line NSO, and others), insect cells (baculovirus-infected *Spodoptera frugiperda* cells), and plant cells (tobacco, *Arabidopsis*, rice). The particular HCP profile is unique and specific to the particular host cells under specific culture conditions and manufacturing processes. HCPs can vary in pI (~3–11) and hydrophobicity, and HCPs display a wide range of molecular weights (from ~5 kDa to at least ~250 kDa), depending on the host cell and manufacturing process used. The number of HCPs in upstream samples can run anywhere from several hundred to more than one thousand proteins, depending on the host cell and culture conditions. Although many cellular hosts have been used in biopharmaceutical manufacturing historically, the most experience has been gained using *E. coli* and the mammalian cells CHO, NSO, SP2/0, and human embryonic kidney cell line HEK293. The guidance in this chapter draws most heavily from the experience with these expression systems; however, general principles apply broadly to any host cell system.

In mammalian cells, the recombinant protein is typically secreted from the cells into the cell culture fluid (CCF), along with many of the HCPs. However, it has been observed that intracellular protein trafficking may not proceed in a normal fashion in production cultures. For example, proteins usually associated with intracellular organelles, such as lysosomes, may be found in the CCF of largely viable cell cultures, because the clones have been selected for maximum protein export. In addition, as some of the cells die, their soluble, intracellular proteins are released into the CCF. Some harvest operations also lyse cells; therefore, the resulting harvested CCF typically contains both secreted and intracellular HCPs. While this mixture of proteins incubates in the fermenter, additional changes in the HCP population may occur, for example, as the result of enzymatic activity (e.g., proteinases or sialidases).

HCP assays provide important information about the composition of the material entering the downstream recovery process and how each purification step affects HCP clearance. In some cases, HCPs can even bind to, and co-purify with, certain products. Process characterization and validation studies are needed to show which process steps remove HCPs and also to demonstrate the robustness of these steps for consistently removing HCPs. As such, HCP assays are an essential part of purification process development and help ensure manufacturing consistency. Lastly, reproducible and reliable HCP assays may be required to measure residual HCPs remaining in the drug substance (DS) used to make drug product (DP) that is delivered to the patient. HCP levels should be measured in: 1) preclinical lots used in toxicology assessment, 2) all lots during clinical development, and 3) process validation samples from the final manufacturing process. After approval, HCP monitoring may be required as an element of the control system. Subsequent sections of this chapter discuss in more detail the use of HCP assays in process validation and in a good manufacturing practices (GMP) control system.

2. TERMINOLOGY

To help establish a common nomenclature in the literature and with regulatory agencies, *Table 1* lists common terms with their definitions (indicating how they are used in this chapter) in addition to synonyms that have been used historically. Note that the term "platform" indicates that the same set of standards and reagents is used within a company to test a variety of products made from the same type of expression system (e.g., CHO cells) grown under similar upstream conditions. In the case of platform HCP assays, the antibodies to HCP are obtained from animals immunized with HCP antigens generated from a common upstream process that is applicable to many products, even if the downstream purifications are different. This approach allows the knowledge from prior products to be leveraged. Justification that an assay is suitable for a new product, using the same expression system and common upstream conditions, is therefore often relatively straightforward.

The HCP immunogen used to generate platform anti-HCP antibodies and used often as the assay calibration standard is, by design, comprised of a broad set of HCPs. In contrast, the qualifier "process-specific" indicates that the immunogen/standard has been prepared from a set of HCPs unique to a given process (either a unique upstream cell culture process or a unique downstream purification process). Process-specific assays are, therefore, limited in their utility, and each must be fully qualified for each process. Process-specific immunogens and calibration standards are, by intent, more narrow and specific to a given process. "Commercially available" assays produced by vendors are often derived from a combination of strains and harvest/purification procedures, and these assays are intended to have a broad application; but these commercially available assays are

not specifically designed for a given manufacturer's proprietary cell line, and users do not have control over reagent availability and lot-to-lot consistency.

Table 1. HCP-associated Terminology

Term	Definition	Historical Synonyms
Commercially available	Available to the public for commercial sale; typically a combination of upstream isolates and corresponding antibodies made by the vendor and sold as reagents or kits.	Generic
Platform	The same set of an HCP standard and antibodies is developed with a company's proprietary host cell strain and used broadly within a type (e.g., CHO) across several products when the upstream conditions are similar.	Custom, in-house, proprietary
Upstream process specific	An assay designed from material where the upstream culture process deviates significantly from the platform. This is generally before any purification and may be applied to more than one product if these parameters are similar.	Custom, in-house, product-specific, proprietary
Downstream process specific	An assay designed from materials where the downstream unit operations are used to enrich the HCP population. This may be applied to more than one product if these parameters are similar. This is rarely used today and is not recommended except for certain products with exceptional downstream processing.	Custom, in-house, product-specific, proprietary
Assay for an individual HCP	An assay using a standard composed of an identified, single, known HCP and its specific antibody/antibodies.	Single analyte assay or Custom, HCP-specific
Coverage	Describes the assessment of how completely a population of polyclonal antibodies recognize the population of HCPs. The coverage assessment may be made on the HCP population used as the HCP antigen or from the product production culture.	
Qualification	Demonstration of suitability of analytical methods (including reagents used in these methods) for their intended application to a given process and in-process samples.	
Protein A/G-affinity purification	Affinity purification of antibodies with immobilized Protein A or G[a]	Affinity chromatography
HCP-affinity purification	Affinity purification of antibodies using immobilized HCP (antigen)[a]	Affinity chromatography, immunoaffinity chromatography
Null cell	The cell strain used for production that does not contain the product-specific genetic elements; includes untransfected parental cells and cells transfected with the expression vector but without the product gene.	Parental, blank, or mock-transfected cell
ng/mg	The numerical quantity (ratio) of HCP per product, where ng represents HCP mass and mg represents the product mass. It is calculated by dividing the HCP concentration (ng/mL) by the product protein concentration (mg/mL).	ppm[b]

[a] In some cases, both purifications are performed, typically the protein A/G first, then the HCP affinity.

[b] Although ppm has been used historically, it is not advised because this term is used to reflect mass per unit volume for other types of tests. It is recognized that ng is used conventionally as a value derived from interpolation from an HCP standard curve (in units of ng/mL), where the signal is reflective of antibody binding and, unlike the therapeutic protein concentration measurement, does not strictly reflect the mass of HCP that may be present.

3. HCP IMMUNOASSAY METHODS

Immunoassay methods rely on antibodies that recognize, as broadly as possible, the population of HCPs entering the downstream purification process; therefore, the sandwich immunoassay, designed with polyclonal antibodies, is the workhorse of HCP monitoring and quantitation. This assay format offers a combination of high sensitivity, specificity, throughput, automation potential, rapid turnaround, quantitative results, and low cost per assay that is unmatched by any other currently available assay technology. Other immunoassay formats (e.g., competitive immunoassays) may or may not be suitable, because they lack either the specificity or the sensitivity afforded by the sandwich format. Although these methods result in a single HCP value for a given lot, the number can give greater weight to HCPs for which high-affinity antibodies are present in the reagent(s)—and no or low weight to HCPs which are either not recognized or recognized by low-affinity antibodies in the assay. For these reasons, orthogonal measures of product purity are often needed. More details on these methods can be found in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*.

The basic principles and design of immunoassays are discussed in *USP* chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* ⟨1103⟩. The format that is most commonly used for HCP testing is the sandwich immunoassay with detection systems such as colorimetric, electrochemiluminescent (ECL), chemiluminescent, radioactive, or others. Homogeneous immunoassays, including competitive assays, where all of the reagents are combined at once and the binding occurs in a single step without washing, may be problematic due to antigen excess leading to antibody insufficiency issues (discussed later in the chapter); therefore, these formats should be used with caution. The heterogeneous sandwich immunoassay format described in chapter ⟨1103⟩ is generally preferred, because the dynamic range and sensitivity may be reduced in the homogeneous format. The formats, with their advantages and disadvantages, are discussed further in chapter *Immunological Test Meth-*

ods—General Considerations ⟨1102⟩. Data analysis is typically performed with a nonlinear fit of the sigmoidal curve generated by a wide range of standard concentrations, although some analyses may focus on the low end of the curve for greater sensitivity.

3.1 The Assay Development Cycle

Figure 1 illustrates common assay development plans, depending on the reagents available at various stages. Fewer bridging studies are required when: 1) platform reagents are available, and 2) upstream processes are historically consistent. *Figure 1A* illustrates a scenario where platform methods are not available, and a commercially available assay is used up to the stage of process validation with appropriate assay qualification. For phase III and beyond, either a platform or upstream, process-specific method is preferred. A bridging study should be performed to support assay replacement. If the commercial assay is intended for phase III and post-approval, care must be taken to fully demonstrate that the assay reagents are applicable to the process HCPs. An additional consideration is that the reagents are from an outside vendor over whom the biopharmaceutical manufacturer has less control.

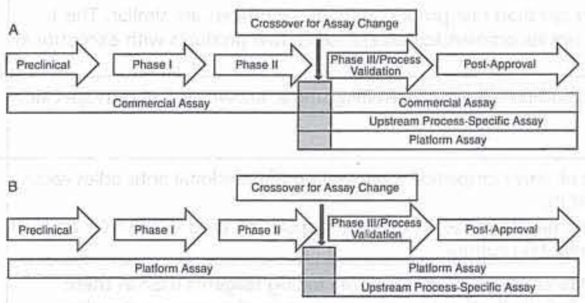


Figure 1A. If there is no platform assay before product development starts.
Figure 1B. If there is a platform assay before product development starts.

Figure 1B suggests that a platform assay can be used through all stages of product development if already available when product development starts. The platform assay should be qualified for each new product. Switching to an upstream, process-specific assay may be necessary for phase III or process validation and beyond, if the cell culture process is significantly changed from the platform process, and may introduce significantly different HCP populations. A bridging study should be performed to support assay replacement.

The limitations of a downstream-specific HCP assay should be considered, because it may be tempting to think that HCP assays are improved by taking the HCPs from null expression through the first column(s) and immunizing animals with a downstream column pool. Historically, the logic was that the immunogen and standard would be enriched in those HCPs most likely to enter the recovery process and be in the final product. This strategy was based on the assumption that, rather than having thousands of irrelevant proteins in the immunogen, only those most likely to be in the process will be present in the immunogen; thus, the resulting assay will be focused on those HCPs of greatest interest. Potential concerns that may arise as a result of this approach are:

1. If only HCPs from a null cell run pool from the first column are used, then later changes during process development (e.g., changes in column run conditions) could invalidate the HCP assay. Thus, process development becomes very restricted and involves the risk of needing to develop multiple HCP assays for slight process changes (or the need to manage the uncertainty).
2. HCPs that co-purify with a product may do so because they bind directly or indirectly to the product protein. A null cell run of a column without the product protein would miss these HCPs.
3. Nonspecific adsorption to chromatography resins is not uncommon and, often, is not a reproducible phenomenon. Compared to the first passage, subsequent passage of the null cell run material over a column will likely produce a different set of HCPs after passage over a new column resin.

3.2 Development and Characterization of HCP Reagents

3.2.1 PREPARATION OF ANTIGEN/HCP STANDARD

As described in *1.1 Considerations for Manufacturing, Characterization, and Consistency*, the total HCP "antigen" is a complex population of proteins; therefore, when generating the HCP antigen/standard reagent, it is important to ensure that: 1) the calibration standard is representative of the cell line and manufacturing process, 2) its protein concentration is accurately quantified, and 3) the immunogen is administered in a way that generates polyclonal antibodies with reactivity to as many different HCPs as possible. The HCP antigen composition should also be comprehensive enough to tolerate normal process manufacturing changes during the life cycle of the product(s).

In addition to the uses above, the HCP antigen may also be needed to prepare the affinity column for purification of the antisera. If affinity purification is used, the quantity of HCP antigen needed should be carefully planned. The amount produced should be large enough to provide sufficient inventory for many years (often 10–20 years); ideally, for the whole life span of the product. However, the antigen preparation process should be performed and documented in a way that facilitates a potential resupply.

Because the HCP assay is used to test DS samples that contain trace HCP impurities, any cross-reactivity of the anti-HCP antibodies with the product may compromise the test method and yield biased results. Therefore, any contamination of the HCP antigen with product must be avoided to prevent the generation of anti-product antibodies.

3.2.1.1 Preparation of HCP antigen from mammalian cells: The majority of biopharmaceutical products produced today are expressed in mammalian cells, e.g., CHO cells. A typical process for the preparation of CHO HCP antigen is outlined in *Figure 2*.

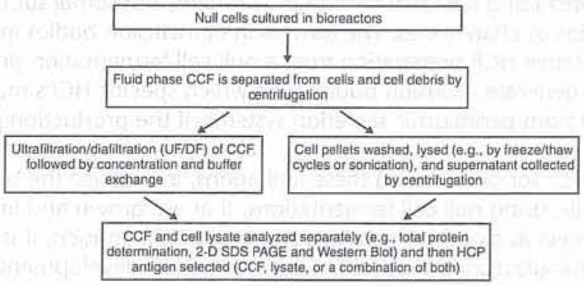


Figure 2. Example of mammalian HCP antigen preparation.

The first step is to establish a null cell that does not express the product gene, either by using non-transfected (i.e., parental) cells with a common origin to those used to make product alone, or to transfect them with the vector used to create the production cell line without the product coding gene (historically described as a "mock" transfection). The main advantage to the latter is that selective markers (e.g., dihydrofolate reductase or glutamine synthetase) are expressed. Alternatively, antibodies can be raised to selective markers independently. Another advantage is that mock-transfected null cells express selection markers and may be grown in cell culture conditions more closely resembling the manufacturing process. Using pools of mock transfected null lines which have not been cloned allows for the maximum potential genetic diversity and therefore gives the highest potential of generating a broad host cell protein population. However, these cultures, which are seeded with cell pools (not cloned), may demonstrate more variability from run to run. Both approaches [non-transfected (parental) and mock transfected] are commonly used. Both are also subject to the following issues: the cell culture process optimized for the production clone may not lead to similar viability and cell density of the null cells. In addition, in the absence of the stresses experienced by cells producing large quantities of product protein, the null cell lines may exhibit a different HCP profile. These changes in viability, cellular metabolism, and cell density may alter the HCP profile of the antigen, making it less representative of the manufacturing process. More about choosing appropriate HCP antigen preparation conditions is discussed below.

Once the null cells have been established, the HCP antigen is prepared in a bioreactor, cell culture flask, or cell culture bag reflective of the product or process cell culture conditions. A platform cell culture process may be applied, which represents a common manufacturing process for several products (e.g., CHO-derived monoclonal antibodies). Typically, the resulting HCP antigen is minimally processed to ensure a broad HCP spectrum, making the antigen suitable for HCP monitoring of multiple products generated from the platform process. A platform HCP antigen also may be produced by combining several null cell runs with slightly different cell culture conditions (e.g., allowing the culture to remain in the bioreactor for longer periods of time to vary the cell viability). This approach is used to obtain a broader HCP spectrum, and antisera raised against this immunogen may be suitable for HCP detection in products generated from a variety of slightly different processes. These reagents also may increase the robustness of the HCP immunoassay toward process changes.

Alternatively, the HCP antigen may be prepared using an upstream, process-specific approach in which the cell culture process is tailored to mimic the production process for a unique product or specific process. The advantage of this approach may be the high relevance of the resulting HCP antigen to a particular upstream process. As with platform reagents, because no additional processing purification steps are included, the antigen contains a broad range of HCPs, and the antigen will often remain suitable for HCP monitoring after downstream production-process changes. The disadvantage of this approach is that the use of the reagents typically will be limited to a single (or few) product(s) or process(es).

The HCP antigen can be produced from a representative small scale, pilot scale, or production scale run. There are pros and cons for each approach. Pilot or production scale may mimic the actual process best; however, normal variation between cell culture runs may not be reflected if only one run at large scale is applied. Conversely, several small-scale preparations can be pooled and may better reflect the run-to-run variability of the cell culture process. Regardless of the scale, the presence of product should be aggressively avoided, because its presence in the HCP immunogen will compromise the quality of the resulting immunoassay antisera.

After performing the null cell run, the HCP antigen can be prepared from the cell lysate, the concentrated CCF, or as a mixture of both as shown in *Figure 2*. If the antigen is prepared from CCF, the cells may be harvested at a time when production

line harvest is performed or some days later, to allow for some of the cells to lyse and release HCPs into CCF and thereby broaden the HCP spectrum.

To prepare immunogen from cell lysate, the cells are harvested by centrifugation, and the cell pellets are washed and lysed (e.g., by using repeated cycles of freeze/thaw, or high pressure homogenization). Conditions are typically selected to mimic the production process (i.e., to prepare immunogen from CCF, cells and debris are removed by centrifugation, and the CCF is then treated by ultrafiltration/diafiltration). The buffer is exchanged (e.g., into PBS or HEPES), and the CCF is concentrated. The cut-off for filtration should be chosen to minimize loss of HCP, e.g., 10 kDa or less.

3.2.1.2 Preparation of HCP antigen from bacterial cells: Many of the above-described principles used in the preparation of mammalian HCP antigens also apply to prokaryotic cell culture. However, some unique challenges should be considered. In particular, manufacturing processes for bacterial expression system come in more varieties, such as those yielding concentrated protein deposits known as inclusion bodies, which can represent up to 95% of the total cell protein. HCP impurities are also present in the inclusion bodies, including bacterial membrane proteins, ribosomal subunit proteins, and cytoplasmic proteins, such as small heat shock proteins or chaperones. The formation of inclusion bodies in the production strain makes it more difficult to generate a representative HCP preparation from a null cell fermentation process. The null cell may not have the same HCP profile, and it will not generate inclusion bodies with which specific HCPs may co-purify, as described above. In other cases, HCPs have been created from periplasmic secretion systems, if the production process can be replicated in the null cell.

Because there is no obvious approach for overcoming these limitations, in practice the bacterial HCP antigen is usually generated from the lysates of washed cells, using null cell fermentations, that are grown and induced under conditions representing the upstream manufacturing process as closely as possible. As for any HCP antigen, it is necessary to demonstrate that the HCP profile is representative of the manufacturing process before use in assay development. This approach yields the full complement of HCPs expressed and hence is very broad.

Because most animals have been exposed to bacterial antigens and may have pre-existing antibodies to many bacterial cell proteins, it is important to characterize the pre-existing antibody responses, and if appropriate, consider the use of specific pathogen-free (SPF) animals. The presence of significant levels of pre-existing anti-HCP antibodies may confound the analysis of the antibody responses; therefore, it is important to screen preimmune sera from animals for pre-existing antibodies prior to immunization.

Null bacterial cells transfected with the expression vector contain chaperone genes that may be expressed at high levels (e.g., 5% of the total HCPs). Such overexpression of particular HCP impurities might necessitate the development of single-analyte assays for these particular impurities (see *6.1 Assays for Individual HCPs*).

3.2.1.3 Characterization of the HCP antigen: Several analyses are recommended before immunization: 1) protein content (total protein assay); 2) absence of product (as shown by Western blotting, immunoassay, and/or MS analysis); and 3) characterization by 1-D or 2-D-polyacrylamide gel electrophoresis (PAGE). Protein concentration is measured to establish the standard concentration for future use and to determine the overall amount of the prepared antigen. The most commonly used methods are the bicinchoninic assay (BCA), the Bradford assay, and amino acid analysis (AAA), although the colorimetric methods require a standard (e.g., BSA) that is not well matched to the (HCP) analyte. Absorbance at 280 nm (A280) may also be used although it is less specific for protein (e.g., nucleic acids will also be measured). These methodologies often provide similar results, but some methods may be more significantly affected by the presence of interfering substances than others. One should consider using two orthogonal methods to exclude a gross over- or underestimation of the protein concentration by one particular method. For more information, see general chapter *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩, which discusses the advantages and disadvantages of various protein analysis methods. The HCP concentration must be assigned with a scientifically sound approach that is used consistently for its lifetime. The lack of product can be assessed with gels, anti-product Western blots, or other suitable methods. Lastly, the 1-D and 2-D gels help characterize the pattern of HCPs, show that a broad spectrum of proteins is present, and show that a reasonable match to production is present. However, this can be challenging because of the presence of large amounts of the product, which can obscure HCP detection. Comparisons may be made between the specific populations of host proteins produced in the null cultures and production cultures using the orthogonal methods discussed in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*.

3.2.1.4 HCP standard reference reagents: Frozen HCP material is usually stable for a very long time, but stability of the HCP standard reagents should be monitored over time because the assay lifetime can be very long. HCP standard stability can be confirmed by monitoring the HCP standard performance in the HCP immunoassay as well as in orthogonal methods.

Appropriate controls within the assay range may be established to monitor assay performance. Controls may be prepared from independent dilutions of the HCP standard, product samples or intermediate pools, or spiked product samples. However, there is a slight risk that control samples prepared as a dilution from the same HCP standard material can degrade at the same rate as undiluted HCP standard, and degradation of the standard will not be detected. To mitigate the risk, data from several standard curve parameters (e.g., signal, background, slope, coefficient of determination) are often assessed for each assay and used to support HCP standard stability. Using material different from the reference to prepare controls will help ensure that the degradation rate will be independent, and HCP standard degradation can be detected easily.

Assay control charts can be established and used to record reference curve parameters, control sample values, and lot-specific information for the critical reagents, such as labeled HCP antibodies. Acceptable ranges for the controls should be established on the basis of multiple runs (usually 20–30) and may be used as part of routine assay acceptance criteria. In addition, the values for % coefficient of variation (CV) of standard and control replicates are often a part of the assay acceptance criteria.

Differences over time in HCP standard curve performance or HCP control levels, or an increase in assay variability, may indicate a lack of HCP standard stability.

3.2.2 PREPARATION AND CHARACTERIZATION OF ANTIBODIES

3.2.2.1 Preparation of antibodies to HCPs: The anti-HCP antibodies prepared are often used for many applications (Western blots, immunoassay) over a long period of time (e.g. the anticipated life of a product or platform). Therefore, material needs should not be underestimated. The recommended antisera quantity will depend on many factors including the intended use, the predicted quantity purified from a given volume of antisera, the amount coated on microtiter plates, and many other considerations. The choice of animal species used to generate anti-HCP antibodies is driven by the immunogen, the requirements for the particular method, and the preferences of the individual investigator. Rabbits, sheep, and goats are often chosen for immunization programs. In some cases, multiple animal species have been used. In such cases, using species more phylogenetically distant from mammals (e.g., chickens) have helped to generate anti-HCP antibodies to conserved mammalian HCP proteins. The choice of animal species may also be driven by a desire to either increase the amount of antisera available from one or several animals (goats or sheep), or to use a strategy to obtain a greater diversity of responses provided by the use of many more individual animals from a single species (e.g., rabbits). The number of animals chosen to generate the antiserum pool(s) depends on the species used and the duration of the immunization protocol. If smaller animals are used to generate the antiserum, more individual animals are generally needed (e.g., 10–20 rabbits). In contrast, if larger animals (goats or sheep) are used, investigators usually will immunize 3–10 animals per protocol. Typical immunization protocols provide approximately 100–150 mL of antiserum per rabbit or approximately 500 mL of antiserum per goat, and the total yield of Protein A or Protein G purified antibody is approximately 1 gram per rabbit or 5 grams per goat. Animals should be screened for pre-existing antibodies against drug substance prior to immunization. If positive results are found, then these animals should be excluded from immunization.

A portion of the prepared HCP antigen is mixed with an appropriate adjuvant (most commonly, Freund's adjuvant or in combination with incomplete adjuvant) and used for the immunization of animals. Each animal receives an initial priming immunization, followed by multiple (often 4 to 8) booster immunizations, to mature the immune response and generate high-titer, high-affinity antibody responses. Antiserum is collected from each animal before the initial priming immunization (time 0) and at appropriate intervals (usually 10–14 days) after booster doses. Four to six bleeds are usually collected from each animal. However, the duration of the immunization protocol may be extended by increasing the number of booster immunizations, making it possible to collect additional high-titer antisera. Other immunization strategies may also be helpful (e.g., cascade immunization or size fractionation of the HCP immunogen).

Investigators should perform titer or Western blot analyses of the individual test bleeds before antiserum pooling to screen for and remove sera that 1) have low titer, 2) display an immunodominant immune response to a small group of HCPs, or 3) have nonspecific binding characteristics or anti-product reactivity. This evaluation should also be performed on the final antibody pools to demonstrate broad coverage of HCPs and a lack of binding to the therapeutic product. In addition, some laboratories have found it useful to evaluate antibodies for aggregates using size exclusion chromatography as described below. Acid elution conditions may cause some antibodies to aggregate, and multimers of this type, particularly when labeled with either biotin or horseradish peroxidase, may lead to increased assay background. In addition, some laboratories include a final process-scale size-exclusion chromatography step to reduce aggregate levels to low levels (e.g., <5%).

Multiple approaches to the purification of antibodies from antiserum pools have successfully generated antibodies suitable for use in HCP immunoassays. Antibody purification is often performed by Protein A, Protein G, or HCP column affinity chromatography. The application of coated magnetic beads can also provide benefits in some scenarios. The choice of Protein A or Protein G is driven by which animal species was used to generate the antibody. Manufacturers provide standard protocols for these routine antibody purifications. When affinity purifying anti-HCP antibodies, materials such as chromatography resins, which may have been used previously for other products, should be avoided, and additional cleaning cycles should be in place to minimize carryover. Ideally, manufacturers should use a resin that has never been exposed to a DP. Before the affinity purification step, some laboratories also include an initial 50% ammonium sulfate precipitation of the crude antiserum to enrich and concentrate the antibody fraction and thereby extend the performance and duration of use of affinity columns. The use of Protein A or Protein G purification strategies generates reagents in which the anti-HCP-specific antibodies compose a smaller proportion of the total antibody. In contrast, HCP affinity purification strategies selectively purify anti-HCP-specific antibodies from the antisera or antibody-enriched preparations. In this case, the HCP antigens are coupled to an activated resin, and the antiserum is purified over the column following established protocols or the resin manufacturer's instructions. The purified antibodies may be further purified by size-exclusion chromatography to remove aggregates, then dialyzed, concentrated, divided into aliquots, and stored frozen (typically −70° or colder). Neat antisera should also be stored frozen (see 〈1106〉 for additional information regarding storage of serum samples).

Affinity purification of specific antibodies using HCP immobilized on a column requires careful management of the column preparation and use so that it can be reproduced reliably for future purifications. In addition, if reused, appropriate resin storage and regeneration procedures should be evaluated and controlled to avoid degradation during storage. Done properly, it is a reliable, reproducible process that has led to consistent anti-HCP antibodies and HCP immunoassays. After loading the column with antisera, an optimized wash procedure is critical. Because the preparation is a mixture of antibodies with differing affinities, extensive washing may remove low-affinity antibodies. Similarly, loading large amounts of anti-HCP antisera onto the

column may result in high-affinity antibodies, displacing low-affinity antibodies if they recognize epitopes that are in close proximity. In either case, the low-affinity antibodies may be removed. In theory, higher affinity antibodies may also be more challenging to elute. Whatever load and wash conditions are initially selected for the antigen-specific affinity purification, these conditions need to be maintained in future production lots of the antibodies. A comparison between these two purification strategies is shown in *Table 2*. Both approaches are commonly used.

Table 2. Antibody Purification Strategies

Type of Purification	Pros	Cons
Protein A or G Affinity Column Chromatography	Robust and reproducible for quickly separating IgG fraction from other serum proteins	Lower proportion of antibodies specific to HCPs and may have a higher amount of low affinity antibodies
HCP Affinity Column Chromatography	Enriches for specific high affinity anti-HCP antibodies; excludes nonspecific antibodies and serum proteins	More skill and documentation required to make and maintain resins consistently. Some high affinity anti-HCP antibodies may not be eluted in usable form and may not be recovered

3.2.2.2 Characterization of antibodies to HCPs: Antibodies prepared for capture and detection are assessed independently and as part of the sandwich immunoassay pair. The concentration of the unmodified antibody is determined most commonly by absorbance at 280 nm, AAA, or BCA. Each approach is acceptable, provided it is applied consistently and (if colorimetric) is standardized similarly. It is important to determine the concentration of the capture and detection antibodies in each batch because they will be diluted to a certain concentration in the optimized immunoassay method. The detection and capture antibodies are characterized by their performance in the immunoassay method(s). The best label to antibody conjugation ratio should also be empirically evaluated primarily based on the best performance in the assay. This optimal ratio should be targeted in the future when new labeled antibodies are made. Finally, although antibodies are generally very stable when stored frozen (e.g., −70°), investigators should ensure storage integrity and determine a defined period of use. This can be accomplished by continuous monitoring with trending charts or by requalification experiments at specified time intervals.

The quality of the antibody pair (capture and detector) is often evaluated in two ways:

1. Show that the antibody pairs are specific and sensitive in an immunoassay format for the HCPs present in a series of samples taken from the unit operations of a given purification scheme, including the DS. This topic is also addressed in more detail in *4. HCP Immunoassay Method Validation*. In concept, the ability to detect and measure HCPs (immunoreactivity) in a series of actual process samples from a given process for a product in development is demonstrated with data showing: 1) sequential clearance as the product is purified, 2) sample linearity throughout a dilution series, and 3) lack of cross-reactivity to product or matrix. The reduction of HCPs during the purification process is determined by clearance factors where the HCP content (in ng/mg) at each step in the process is divided by the amount in the prior step. Typically, starting samples from the cell culture may have HCP levels ranging from several hundred thousand to several million ng/mg, and final products may have HCP levels ranging from <1 to 100 ng/mg (showing many logs of clearance). When these trends are observed, they suggest that the process and the assay are functioning effectively, and the antibodies used in the immunoassay have suitable quality attributes. If these trends are not observed, it may not necessarily reflect a problem with the antibodies, because it is also possible that the purification process is not effective.
2. Show that a broad range of HCPs in the calibration standard is recognized (i.e., that the coverage of the HCP population is adequate). Coverage is evaluated for at least the capture antibodies using 2-D gel Western blots or immunoaffinity fractionation of the total HCP population by immobilizing the anti-HCP antibodies on a column. In addition it is recommended to test the coverage of the detection antibody if different coating and detection antibodies are used. The benefits and limitations of using either 2-D gels or immunoaffinity fractionation to demonstrate coverage is discussed more fully below. Whichever method is used, the extent of coverage must be addressed in qualifying the immunochemical reagents.

HCP coverage evaluations help assess the ability of the antibodies to recognize a wide range of HCPs in the calibration standard and those present in in-process and DS samples. Two methods (2-D gels followed by Western blot analysis and immunoaffinity purification followed by 2-D gel analysis) are in fairly common usage, and both have the limitation that they tend to underestimate the true antigen binding in immunoassay methods but may have value in comparing two preparations head-to-head (or a platform to a commercial reagent). Both procedures use reducing 2-D gel electrophoresis to separate HCPs in the HCP standard or in early process sample(s) (e.g., harvest). Numerical coverage comparisons should be used with caution because of the many method variables and the art required to reproduce results, even with the same reagents in the same laboratory. Because of this variability, the results are best evaluated qualitatively. Comparisons of batches (or sources) of antibodies should be done side by side as much as possible to determine if antibody lots are comparable or if one is superior to another. One approach, the SDS-PAGE/Western, is essentially a comparison of the number of spots present in the immunologically stained membrane versus the number in a duplicate gel (or blot) stained for total protein (e.g., by fluorescent dye or silver; see also *USP* chapter *Immunological Test Methods—Immunoblot Analysis* ⟨1104⟩). Differential staining of each antibody preparation may power the analysis, because it can be used to analyze the same 2-D gel. The second approach, immunoaffinity binding/SDS-PAGE, involves comparing the flow through and eluate to the load from the HCP calibration standard (or early process sample) passed over a resin to which the capture antibodies have been covalently immobilized. The resin is washed after loading and before elution to remove nonspecifically bound HCPs. For the purpose of sample comparison, difference gel electrophoresis (DIGE) technology could be helpful. *Table 3* lists the advantages and disadvantages of these two coverage methods.

Table 3. Comparison of Coverage Methods

	Pros	Cons
2-D SDS-PAGE/Western blot	Good separation of individual HCPs allows for individual spot counting.	HCP antigens are denatured, may not represent what is seen in immunological assays (e.g., ELISA).
		High variability—numerical "percent coverage" values vary widely with the same material tested within a single lab and between different laboratories.
		Transfer efficiency of a broad range of HCPs difficult to optimize, leading to underestimates due to over-transfer through the membrane or failure to transfer from the gel, dependent on molecular weight.
		Matching spots between blots and gels are difficult and not standardized.
Immunoaffinity binding/ 1- or 2-D SDS-PAGE	HCPs bind to antibody resin in solution under native conditions similar to the sandwich immunoassay.	May underrepresent some HCPs if they are bound too tightly and do not elute, resulting in an underestimation of coverage.
	Analysis of spots in gels does not require immunoblotting and is simpler because the problems of transfer are avoided.	Preparation of the anti-HCP resin must be done carefully and may be difficult to reproduce. Results can be dependent on resin loading and elution conditions.
		Proteins at low concentration that only show up in Westerns will not be detected.

More information about the appropriate use of SDS-PAGE/Western blot methods and ways to minimize the disadvantages above can be found in the later section *5.2 Considerations for Western Blot Methods*.

An example of a suitable use of this approach is shown in *Figure 3*.

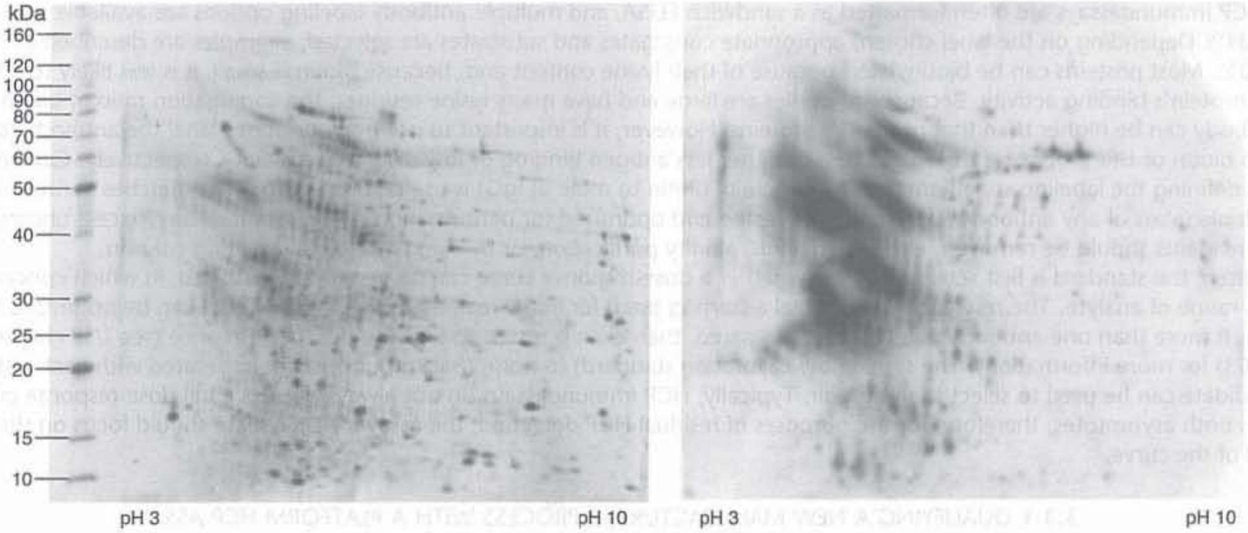


Figure 3. Left panel: 2-D IEF/SDS-PAGE analysis of representative CHO HCP calibration standard stained with a sensitive fluorescent stain. Right panel: Western blot analysis of the same gel shown in the left panel.

3.2.3 GENERATION AND QUALIFICATION OF REPLACEMENT REAGENTS

When the supply of HCP reagents are depleted or if their performance declines, new reagents may be needed. Often, new HCP antigens and new HCP antibodies are both generated at the same time. In theory, the new set of reagents should be created by following the same protocol that was used for the previous set of reagents to match the performance of the new and old assays as closely as possible and produce consistent data for a given program. In practice, however, it is not always possible (nor advisable) to generate the assay reagents exactly the same way that they were originally made. For example, if the manufacturing process has been optimized over time and the original platform assay reagents are no longer relevant, a careful assessment should be made. When such changes are required, the ideal way to confirm the quality of the replaced reagents is through characterization of antibodies (see below) and demonstrating that they can detect similar or greater HCP log reduction, from harvest to DS, using the same samples tested head to head with the original assay reagents.

When replacing an HCP antigen in which the current production process is essentially the same as the old process, the new HCP antigen should be prepared as similarly to the old as possible. It is important to compare the protein concentrations of the old and new HCP antigens side by side, using the same method to correctly assign the protein concentration for the new

antigen and to ensure that the protein concentrations are comparable in the new and old HCP calibration standard. Characterization by 1-D and 2-D SDS-PAGE should also be performed as a side-by-side comparison of the old and new HCP antigens (see above) to be sure that gross changes in protein composition have not occurred.

For antibody replacement, the same animal species should be used, if possible. In cases where a better antibody response is desired, antibodies produced by different species may be evaluated. The selection of the species should take into account all the points discussed in this chapter. A side-by-side comparison of results from process samples using old and new antibodies in a sandwich immunoassay format should be included to demonstrate suitability of the replacement antibodies. Additional characterization by orthogonal methods (e.g., coverage using 1-D and 2-D SDS-PAGE) is recommended, as described above. Assay qualification or validation should also be considered after changing reagents.

To fully understand the effect of replacing HCP critical reagents on assay results requires testing of process samples in bridging studies using multiple samples from harvest, process intermediates from all appropriate purification steps, and the final DS. These studies can be very resource intensive, which underscores the importance of making sufficient quantities of HCP critical reagents at the outset (and minimizing replacement).

3.3 Immunoassay Method Development and Qualifying as Fit for Use

As noted in *1. Introduction and Scope*, HCP immunoassays serve two important functions in the development and control of biopharmaceutical manufacturing processes. HCP immunoassays serve as a measure of product purity, and therefore are potentially related to patient safety. They also serve as a measure of manufacturing consistency, and therefore they are reflective of process control. To meet these requirements, HCP assays are often validated and incorporated into the cGMP control system, which includes specific reject limits (in the DS as a Certificate of Analysis (C of A) method or as an in-process control). If the final DS is a conjugated protein, the testing should be done on the protein intermediate (before conjugation). In addition, HCP assays are used to guide process development and are often used to demonstrate robust process performance in the context of formalized process validation activities.

HCP immunoassays are often formatted as a sandwich ELISA, and multiple antibody labeling options are available (see ⟨1103⟩). Depending on the label chosen, appropriate conjugates and substrates are selected; examples are described in ⟨1103⟩. Most proteins can be biotinylated because of their lysine content and, because biotin is small, it is less likely to affect the protein's binding activity. Because antibodies are large and have many lysine residues, the conjugation ratio of biotin to antibody can be higher than that of smaller proteins. However, it is important to not over- or under-label the antibody (e.g., with biotin or HRP), because this might lead to either less antigen binding or lower signal/sensitivity, respectively. Controlling and defining the labeling stoichiometry (e.g., mole of biotin to mole of IgG) is useful for making future batches consistently. Multiple ratios of any antibody label should be tested and optimized for performance in the immunoassay. Excess unconjugated reagents should be removed, either by dialysis, affinity purification, or by using a suitable desalting column.

Often, the standard is first screened to see whether a dose-response curve can be generated, and if so, in which concentration range of analyte. The results can also reveal a starting point for initial reagent concentrations that can be optimized further. If more than one antibody pair has been prepared, then each is tested to find the best performance (see *USP* chapter ⟨1103⟩ for more information). The signal (low calibration standard) to noise (background) ratios generated with each antibody candidate can be used to select the best pair. Typically, HCP immunoassays do not always possess a full dose-response curve with both asymptotes; therefore, for the purposes of residual HCP detection, the assay for DS release should focus on the low end of the curve.

3.3.1 QUALIFYING A NEW MANUFACTURING PROCESS WITH A PLATFORM HCP ASSAY

As noted earlier, if the upstream/isolation procedure is matched, then a new product produced in that system can benefit from a platform assay approach that can decrease qualification time and effort. However, in cases where there are variations that occur in optimizing the production cultures and the cell culture design space is not completely defined, it is important to understand whether these variations are significant enough to render the platform assay unsuitable. In these cases, the platform assay should be qualified experimentally to demonstrate that it is suitable for measuring HCPs from the new process. Assay accuracy, precision, sensitivity, linearity, range, and specificity must be evaluated (see *4. HCP Immunoassay Method Validation*). In addition, the range of HCPs in the platform HCP standard should be compared to those present in the new process. The antibodies should also be characterized using samples from the new process or product using approaches discussed previously.

The following approaches and concepts may be useful in determining if a platform assay is suitable for a product made with a new process:

1. Conduct a representative, small-scale null cell run grown under conditions used in the new culture process, and determine the protein concentration of the null cell run CCF. Assay the null cell run CCF at the same nominal concentration as the assay HCP standard using the HCP sandwich immunoassay, and compare the dilution curve from the null cell run CCF to the standard used in the assay. If the standard and new null cell run CCF curves are similar (e.g., in shape and amplitude, and the calculated HCP concentrations are within a factor of two), then the new process is considered not different from the platform, and a process-specific HCP assay is not usually needed.

2. With the CCF from the above null cell run from the new process, make a comparison to the HCP calibration standard using 2-D gel electrophoresis. This will compare the diversity of proteins produced by the two processes. However, be aware that new "spots" may appear in the 2-D gels that may not reflect immunochemical differences in the assay, for example, post-translational modifications or limited proteolysis. As such, the appearance of a few new spots or up-regulated or down-regulated spots in the process-specific null cell run is not a basis for invalidating the application of the platform assay. A qualitative assessment (e.g., versus a Western blot) is recommended.
3. Comparison of HCP levels at harvest in actual production runs (CCF samples) with the same product produced in the original versus the "new" process is also valuable. In general, immunoassay results within a factor of two are often considered similar, thereby confirming the platform HCP reagents.
4. HCPs in CCF from production cultures may also be estimated on the basis of total protein measurements. The total protein in the CCF is the sum of product (measured using a product-specific titer assay) and HCPs. Samples from harvests of the manufacturing run cell culture can be dialyzed, as long as they are sufficiently concentrated to make membrane losses negligible, to remove small peptides and amino acids, and then the total protein is determined (e.g., by amino acid analysis). Subtraction of the product concentration from the total protein provides an estimate of the non-product protein, HCP, in mg/mL. This value may be compared with the HCP concentration from the immunoassay. Comparable values (e.g., within 2×) indicate that the HCP platform is confirmed for the new process.

3.3.2 NEED FOR NEW REAGENTS AFTER PROCESS CHANGES

Major changes, such as substantial and unambiguous changes in cell culture conditions (e.g., moving from a serum-containing process to a serum-free production process), typically require production of new reagents and development and validation of a new HCP assay. In this example, the HCP reagents created using the old process recognize a number of serum proteins but may not recognize many new HCP proteins in the serum-free process. Because of both the process change and the assay change, different HCP levels will be observed when the same samples are measured for HCP in the old and new assays (i.e., the samples from the serum-containing process may have a high level of HCP measured by the old assay and low level of HCP measured by the new assay, whereas the samples from the serum-free process may have much lower HCP results by the old assay but more with the new assay).

If the assay was developed as a downstream, process-specific assay, changes in the purification steps can also require replacing the assay with a new one that is relevant to the new downstream process. This is the biggest problem with these very specialized assays and why platform-based assays are usually preferred, because they often do not require a new assay when the downstream steps are changed.

3.3.3. SAMPLE STABILITY

It is important to demonstrate that the sample handling procedures do not affect the assay results. This is particularly true when comparing results for various intermediate process pools. Occasionally, HCPs can precipitate during freezing or when the sample pH is adjusted from acidic to neutral. In addition, storage at 2°–8° or multiple freeze-thaws may result in loss of reactivity. The analyst may need to add specific stabilizing components (e.g., divalent cations) to the samples to ensure stability.

Change to read:

4. HCP IMMUNOASSAY METHOD VALIDATION

HCP immunoassays for product purity assessment typically are part of the cGMP control system, either as an in-process test or on the C of A, and need to be validated appropriately. The validation approach depends in part on the types of samples that will be tested with the method. All validation parameters for a quantitative impurity test are needed when used for the final DS, whereas validation for in-process samples usually focuses on dilution linearity, interference, and precision. Regardless of whether the assay is an in-process or release assay (DS), action limits are valuable as a part of an overall control strategy. Readers are referred to ICH Q2(R1) guidelines and chapter *Validation of Compendial Procedures* ⟨1225⟩ for general expectations for assay validation, particularly those requirements for cGMP assay validation, and to chapter ⟨1103⟩ for sandwich immunoassays. Although the main focus of this section is on cGMP assay validation, many of the same principles apply to the assays used to demonstrate that process purification steps are suitable for their intended purpose.

This section will focus on those aspects of HCP immunoassays that are unique or require special attention and documentation. HCP immunoassays differ from more conventional immunoassays in that they are multi-analyte assays. There are potentially thousands of proteins in the immunogen and standard, and a correspondingly diverse set of antibodies is produced, yet only upstream CCF will contain a diversity of proteins that approximate the HCP calibration standard. Intermediate pools and final products typically contain progressively fewer of the HCP proteins that are present in the standard. As discussed previously, this is the aspect of accuracy that is compromised by HCP immunoassays, and why the mass unit ratios (ng of HCP per mg of DS) are not a literal "mass" relationship. However, below are common industry approaches that address these limitations as much as possible.

General Chapters

The validation summary below is for the final DS or a penultimate process step, whichever is chosen for the C of A method. It should be noted that for in-process testing, the focus is on method accuracy (spike recovery), dilution linearity, and precision. In the case of accuracy, some samples will have more potential interfering substances (other process impurities or additives) that may need to be confirmed as non-interfering (e.g., residual DNA, leached protein A, virus inactivators, chaotropes, and low pH buffers).

4.1 Accuracy

To assess HCP assay accuracy in the DS, at a minimum the analytical assay standard should be added to appropriate samples and accurate spike recovery demonstrated over the range of the assay. Matrix interference can come from buffer components and product, and the minimum dilution required for acceptable spike recovery (typically between 70% and 130%) needs to be determined for each sample type. Typically, spike recovery experiments are conducted with spikes using at least three and potentially as many as five different levels. Because samples are assayed at multiple dilutions, the investigator may spike and then dilute the sample, or dilute the sample and then spike the diluted samples. Spikes near the quantitation limit (QL) help to evaluate the accuracy and repeatability of the assay near the QL, which is where the measurement is often the most variable. A spike recovery of 50%–200% may be acceptable for a spike at or near the QL.

Although these are minimum requirements for assay validation, they should not be interpreted as demonstrating accuracy for any one specific HCP that may co-purify with the product. To accomplish that, comparison to a standard of that particular HCP species is needed; however, because this HCP is rarely known, this may not be possible. Tips for discerning these situations are provided below in the dilution linearity section and in *5. Supporting Technologies for Residual HCP Detection, Identification, and Measurement*.

4.2 Sensitivity and Assay Range

HCP sandwich immunoassays often achieve standard curve sensitivity in the low single digits of ng/mL. For sample protein concentrations where the test dilution is 10 mg/mL, this implies that a sensitivity of about 1 ng of HCP per mg of product is possible. However, this sensitivity may be difficult to achieve for products with lower protein concentrations. It is important to highlight that the reported HCP ratio (ng of residual HCP relative to mg of product) is not really the ratio of masses implied by the unit ng/mg but rather "immunological equivalents" per mg of product.

The first aspect of determining QL is typically determined experimentally for each product in a given sample buffer matrix in spike recovery studies. The minimum recommended dilution (MRD) for the DS should be established by spiking the HCP standard in a sample dilution series. For example, an HCP spike of 10 ng/mL added to each of a series of solutions containing undiluted (e.g., 10 mg/mL) protein and serially diluted DS. Those dilutions in which the spike recovery is 70%–130% (50%–150% is also commonly used for levels near the QL) are considered acceptable. Typically, a proposed DS MRD is tested further to ensure that spike recovery is consistently achieved.

For the second part of the QL determination, the DS is tested at the MRD (if the DS samples have high levels of detectable HCP, a formulation buffer may be used). The QL is generally determined by the analysis of spiked concentrations of HCP and by establishing the minimum level at which the HCP can be determined with acceptable accuracy and precision. This study is typically performed at least three times, preferably by different analysts or on different days. For example, a spike of 3 ng/mL of HCP in a product protein concentration of 5 mg/mL has a QL of 0.6 ng/mg or 3 ng/mL if spike recovery is achieved in, e.g., at least four of six tests or if mean spike recovery criteria are met.

Typically, assays are set up to measure the range from a few ng/mL to >100,000 ng/mL. This range allows the analyst to test a variety of samples at multiple dilutions (see discussion of *4.3 Sample Linearity*) and allows for a practical assay that can accommodate in-process pools with highly differing HCP levels. For example, upstream samples may contain >100,000 ng/mL of HCPs and require large dilutions to obtain results in range of the standard curve.

For routine commercial DS manufacture where the protein product concentration is known and the process impurities are well understood, testing at a single dilution may be used for release. The results are reported as the ratio of measured HCP (ng/mL) to the product concentration (mg/mL) resulting in units of ng/mg. When the DS has undetectable levels the results are reported as "less than" the assay QL (ng/mL) divided by the product concentration (mg/mL; e.g., <0.6 ng/mg in the example above). Before setting this target concentration for testing, however, the dilution linearity of the samples should be well understood and a robust manufacturing process established. In the event that the level or species of HCPs vary run-to-run it may be necessary to test each sample at multiple dilutions (see below).

4.3 Sample Linearity

HCP assay nonlinearity is not uncommon for some samples and should be assessed for multiple batches of a given sample type (e.g., from several clinical DS lots or process validation batches) to ensure consistency and proper reporting of results. Because a single HCP (or a few individual HCPs) may co-purify with product, it is possible that particular HCP(s) will be present in excess of the available antibodies in the HCP immunoassay. This is possible in multi-analyte assays where only a limited surface area is available on assay microtiter plates or beads, and because thousands of different anti-HCPs are needed, antibodies

to any one HCP will, of necessity, be limited. Furthermore, the relative amount of antibody to each separate HCP is also not controlled, so the binding capacity for each separate HCP differs. For samples exhibiting this behavior, the analyst must dilute the sample into the range of the assay, i.e., to a point where nonlinear behavior is no longer observed because the HCP has been diluted to a concentration where it no longer exceeds the available antibody. In some cases, the sensitivity of the assay becomes limiting, and one never reaches a dilution where the assay result is independent of the sample dilution. In such cases, the highest HCP ratio value (corrected for sample dilution) within the validated assay range should be reported. A detailed example of how to handle nonlinear dilutions arising as a result of antibody insufficiency follows.

Table 4 and *Figure 4* show data for three different samples tested in an ELISA that has a range of 1–100 ng/mL. In this example, all samples were diluted initially to 10 mg/mL (the MRD, where spike recovery had been previously confirmed), and then serially diluted using a twofold dilution series to below the assay QL of 1 ng/mL. Whereas *Sample 1* (diamonds) dilutes linearly over the full range tested, both *Sample 2* (squares) and *Sample 3* (triangles) HCP results plateau at higher concentrations of the sample. Saturation of the antibodies reflects antigen excess, and determination of the HCP value is made by diluting the sample into the range of linear dilution; visually estimated as <2 mg/mL for the squares and <1 mg/mL for the triangles. [NOTE—If large sample dilutions are required to get into the range of the HCP assay, consider making intermediate dilutions to limit dilution-related errors.]

Table 4.[a] **Raw Data for the Graph in** ***Figure 4***[a]

Product	Sample 1			Sample 2			Sample 3		
Conc. (mg/mL)	HCP conc. (ng/mL)	HCP ratio (ng/mg)	% max ratio value	HCP conc. (ng/mL)	HCP ratio (ng/mg)	% max ratio value	HCP conc. (ng/mL)	HCP ratio (ng/mg)	% max ratio value
10.00 (neat)	49 (neat)	4.90 (ERR 1-Apr-2016)	83%	20 (neat)	2.00 (ERR 1-Apr-2016)	<20	3.2 (neat)	0.32 (ERR 1-Apr-2016)	22%
5.00	28.5	5.70 (ERR 1-Apr-2016)	96%	16.5	3.30 (ERR 1-Apr-2016)	54%	2.5	0.50 (ERR 1-Apr-2016)	35%
2.50	12	4.80 (ERR 1-Apr-2016)	81%	10	4.00 (ERR 1-Apr-2016)	66%	1.5	0.60 (ERR 1-Apr-2016)	42%
1.25	7.4	5.92 (ERR 1-Apr-2016)	100%	7.4	5.92 (ERR 1-Apr-2016)	97%	1.1	0.88 (ERR 1-Apr-2016)	61% (ERR 1-Apr-2016)
0.625 (ERR 1-Apr-2016)	3.1	4.96 (ERR 1-Apr-2016)	84%	3.3	5.28 (ERR 1-Apr-2016)	87%	0.9	1.44 (ERR 1-Apr-2016)	100%
0.3125 (ERR 1-Apr-2016)	1.6	5.12 (ERR 1-Apr-2016)	86%	1.9	6.08 (ERR 1-Apr-2016)	100%	<1	<6	NA
0.15625 (ERR 1-Apr-2016)	<1	<6	NA	<1	<6	NA	<1	<6	NA
Reported HCP ratio value with guide and *n*	Guide 1 = 5.2 (*n* = 6); Guide 2 = 5.2 (*n* = 6)			Guide 1 = 5.8 (*n* = 3); Guide 2 = 5.3 (*n* = 4)			Guide 1 = 1.2 (*n* = 2); Guide 2 = 1.4 (*n* = 1)		

[a] Numerical data for three samples (also graphed in *Figure 4*) show differing degrees of saturation of the response at higher concentrations of product (and HCP impurity) tested. Two guides on how to interpret these data (guides 1 and 2) illustrate how the "linear range" of the assay is limited to the most dilute data points shown in *Table 4* and *Figure 4*. Note—Samples are always diluted until the HCP concentration is below the QL of the assay (in this example the QL is 1 ng/mL). Guide 1: All values within 20% of maximum value are averaged. Guide 2: Values are averaged as long as the CV of the values is <20%, removing less diluted samples first. A third guide might also be used that reports the highest value measured above the QL but the suitability of this approach should be very well demonstrated by method validation.

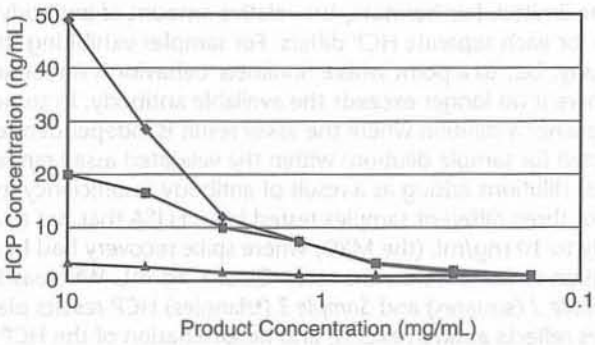


Figure 4. Twofold serial dilution of the three samples listed in *Table 4.* HCP concentration results are plotted against the product concentration in the samples, which serves as a measure of sample dilution.

Because measurements near the QL may have higher variability and less reproducibility than measurements farther from the assay limit, usually (if possible) values within the sample linear HCP concentration response range are averaged. The procedure for which data points may be averaged must be specified in advance of running the assay and is typically documented as part of the test procedure. In the example shown in *Table 4,* two different guidelines are illustrated. *Guide 1* averages all values within 20%–25% of the maximum HCP ratio value. *Guide 2* averages values only if the CV for the resulting averaged values is <20%–25%. Although their data sets are not identical, both rules are justified, based on the argument that variations of 20%–25% are within the variation seen in assay replicates and reflect the underlying reproducibility of immunoassay methods. In this example, both methods give very similar results. In contrast, the third potential guide, which reports the highest value measured above the QL, would yield results that are at least 10% higher. In addition, the validity of the third guide would depend highly on the quality of the method development.

Homogeneous (one-step incubations without washes between reagent additions) sandwich immunoassays are more prone to antigen excess and resulting dilution nonlinearity problems and may exhibit "high-dose hook effects" in which samples with large amounts of antigen give inaccurately low readings, because the antigen excess prevents formation of the complete sandwich. Suitable use of this format should be confirmed experimentally for each sample type and could be acceptable if the samples are diluted to assay QL and lack of hook effects is confirmed.

Historically, some companies have used spike-recovery data produced in the validation of accuracy to demonstrate linearity. Although this demonstrates the linear dilution of the standard, it does not demonstrate the linear dilution of the HCPs in the samples. For the reasons of antigen excess discussed above, the linear dilution of the standard is a necessary condition but not sufficient to demonstrate linearity of the assay for different sample types.

Finally, although antibody insufficiency is generally believed to be the primary cause of a lack of dilution linearity, other potential causes are: 1) reactivity of the antibodies with product protein, 2) the presence of non-specific antibodies ("sticky" antibodies), 3) reactivity of the antibodies to non-HCP proteins present in samples (such as peptones or BSA), 4) HCP aggregation, 5) HCP standard dilution profile that is not representative of the in-process or final product HCP, and 6) sample matrix interference. Cross-reactivity and matrix interference are discussed below.

4.4 Specificity

Assessment of potential matrix interference in samples is addressed in section *4.1 Accuracy* and is primarily used to establish the lack of formulation interference (in the DS) and other impurities that are normally present (for upstream process samples). Another specificity issue to evaluate is the potential cross-reactivity of the anti-HCP antibodies with the product itself. Although this is possible, antibodies to HCPs that cross-react with product epitopes are rarely observed.

In those cases where cross-reactivity is observed, more false-positive assay signals are risks that must be managed. If cross-reactivity is suspected, it should be carefully and scientifically established. In the vast majority of cases, signals in HCP immunoassays are real, indicating the presence of HCP impurities. In particular, instances where the HCP(s) are noncovalently associated with product, and they co-purify, may give the appearance of cross-reactivity. However, because of the tremendous variety of antibodies in a polyclonal anti-HCP pool, when measuring residual HCP in the presence of excessive levels of product, some antibodies may still bind product, yielding a false-positive signal. In this case, it may be necessary to modify the assay or the anti-HCP reagent. If a platform HCP assay is used to test multiple batches (for the same program), and different levels of HCP between batches are observed, this is strong evidence that the HCP-positive signal is real and not due to cross-reactivity.

Evidence for cross-reactivity is often seen in a product that has been purified in a sequence of steps and where the HCP levels are not reduced by typical purification processes. The HCP/product ratio (ng/mg) should be used so that changes in the HCP/product ratio during purification can be compared. First, Western blots of DS are usually probed with anti-HCP antibodies to determine if HCP(s) are noncovalently associated with product. If bands are detected that are unrelated to the product, this suggests that cross-reactivity is not occurring, and further process development may be required if lower HCP levels are sought.

General Chapters

Alternatively, if the anti-HCP is recognizing a product-associated band, this suggests cross-reactivity. Caution should be exercised, however, because the product is denatured (by SDS-PAGE), and binding may be to epitopes not accessible when in the native, solution phase (i.e., in the immunoassay). In this case, a positive Western blot could be an artifact (conversely, a negative Western blot could also miss cross-reactivity, but this is less probable). Regardless, if cross-reactivity is suspected, it can be confirmed and corrected by either: 1) addition of a high-purity product (or related structures, such as human IgG from a different source, for a monoclonal antibody) to block the cross-reacting antibodies in the assay, or 2) depletion of the anti-HCP in the reagents using a product-affinity column made from highly purified product. In either case, it is important to ensure that the antigen used to block or deplete the serum is itself free of residual HCPs. This information is available from the Western blots probed with anti-HCP. If HCP bands are present, this indicates that a higher-purity product may be needed and can be prepared with orthogonal, lab-scale purifications. Sometimes reversed-phase HPLC can be effective in making this material, although this is not useful during product production because of its denaturing properties.

5. SUPPORTING TECHNOLOGIES FOR RESIDUAL HCP DETECTION, IDENTIFICATION, AND MEASUREMENT

Biopharmaceutical manufacturers need an integrated control system that offers assurance of overall product purity and manufacturing consistency. Typically, product purity is demonstrated by using a combination of methods, and this is very much the case for HCPs. In addition to the most common sandwich immunoassay format, other assay types provide valuable complementary information. *Tables 5*, *6*, and *7* provide overviews for commonly used methods for HCP detection, quantitation, and characterization. *Table 5* discusses electrophoretic methods; *Table 6* discusses Western blot methods; and *Table 7* discusses chromatographic and proteomic methods. Gel electrophoresis and immunoblots are also discussed in *3.2.2.2 Characterization of antibodies to HCPs*.

Orthogonal assay methods used to provide additional assurance of purity and the lack of HCPs may be used in any of three modes: "detection", "identification", or "quantitation". In the case of detection, orthogonal assays address the question, "Is there any protein other than a form of the product that the immunoassay may have missed?" If this is the case, as knowledge accumulates regarding the identity of the HCP(s), it becomes possible to perform a specific protein risk assessment and, if necessary, generate a standard and separate test for that particular HCP impurity. The orthogonal method may then be used in a "quantitative" mode by comparing the product sample with the appropriate HCP standard for the known HCP. For example, a quantitative HPLC-MS/MS method may be developed, based on monitoring peptides that ionize well from digests of the analytical standard protein and the sample.

5.1 Considerations for Electrophoretic Methods

Three electrophoretic methods that are useful in impurity analysis are 1-D and 2-D SDS-PAGE and SDS-capillary electrophoresis using non-gel sieving (CE-SDS). In all these methods, excess sample is loaded to ensure detection of trace protein impurities. The drawback is that excess product obscures impurities that migrate close to the product and cause interference. However, 1-D gels or CE-SDS may already be part of the GMP control system for monitoring product-related fragments; therefore, extending these methods for HCP monitoring does not involve additional testing. 2-D gels have been used to investigate the purity of the final product and to identify minor spots in the gels associated with either product variants or HCP impurities. The presence of a new band in the gel, or a new peak in the CE-SDS electropherogram, may represent either a new product-related substance/impurity or may reflect the presence of an HCP impurity ("process related"). In either case, an investigation is warranted. If not part of the routine control system, a 1-D gel is still an excellent method for extended characterization of important lots, such as registration lots, and for manufacturing investigations. Gels and CE-SDS are useful for confirming the absence of gross levels of HCPs (should they be present and missed in the immunoassay). Gels are typically stained with a high sensitivity stain, such as a fluorophore or silver. Ideally, the stain should be compatible with in-gel proteolytic digestion and subsequent analysis by mass spectrometry to identify the protein.

Proteins differ in their staining intensity with silver or fluorescent dyes; therefore, only semiquantitative estimates of the amount of protein in a band or spot are possible (e.g., glycosylated forms may not stain as intensely). Nonetheless, because these methods do not rely on anti-HCP antibodies, they provide an important orthogonal measure of purity.

Some proteins may appear in gels as multiple bands or spots that result from post-translational modifications or proteolytic clipping. To the extent that a single gene product is distributed to multiple locations in the gel, this further reduces the sensitivity of gels to detect impurities.

As discussed following *Table 7* on proteomic methods, it is often possible to excise bands or spots from the gels. These excised gel fragments may then be incubated with proteases to provide *in situ* digestion of the protein into peptides. These peptides may then be fractionated and sequenced using LC-MS/MS and compared with databases to identify the origin of the peptides as either product-related or HCPs.

Table 5. Analytical and Characterization Electrophoretic Methods for HCP Testing

Method	Use	Advantages	Disadvantages	Approximate Sensitivity for HCPs and Ability to Quantify
1-D gel, either reduced or nonreduced, stained with silver or high-sensitivity fluorescent dye.	Screening a large number of samples for consistency and presence of nonproduct protein bands. May be used as part of GMP control system.	High resolution separation; provides approximate molecular weight (MW) information; relatively simple to run. Side-by-side analyses of samples provide powerful comparisons for manufacturing consistency.	Large excess of product protein may obscure HCP bands. Different proteins show differences in staining intensities so only semi-quantitative. Limited capacity of gel lanes for total protein load.	100 ng/mg. Semiquantitative unless used with an analytical standard for a known HCP.
Large-format 2-D gel (e.g., 20 × 25 cm) stained with silver or a high-sensitivity fluorescent dye.	Screening a small number of samples for consistency and presence of unknown protein spots. Not suitable for routine use in lot release but may be used for characterization of specific lots or HCP standards.	High-resolution separation of trace HCP impurities from product. Provides approximate MW and pI information on protein spots.	Large excess of product protein may obscure HCP spots. Different proteins show differences in staining intensities so only semiquantitative. Highly technique-dependent results requiring skilled staff and sophisticated equipment.	100 ng/mg of total protein load). Semiquantitative unless used with an analytical standard for a known HCP.
CE-SDS with laser-induced fluorescence (LIF) detection. Fluorescently labeled samples are separated as either reduced or nonreduced samples.	Screening a large number of samples for consistency and presence of unknown protein peaks. May be used as part of GMP control system.	Rapid, high-resolution method to separate fluorescently labeled proteins.	Sensitivity may not be as high as a 1-D gel with a highly sensitive stain. Fluorescent labels are not specific for HCP proteins, so there is no distinction between HCP and product-related peptides. Difficult to extend to other measures, such as Western blots, or to identify protein peaks.	1000 ng/mg (0.1% of total protein load). Semiquantitative unless used with an analytical standard for a known HCP.

5.2 Considerations for Western Blot Methods

The Western blot technique is complementary to the sandwich immunoassay for demonstrating product purity and for characterizing the immunochemical reagents used in the immunoassay (see *3.2.2.2 Characterization of antibodies to HCPs*). When the anti-HCP antibody is used as a probe, it may be useful to: 1) detect HCPs that are noncovalently associated with the product and (potentially) missed in immunoassay, or 2) monitor an individual HCP. In other cases, a protein that reacts well in the immunoassay may not be detected in Western blot because a conformational epitope was lost following denaturation. Immunoblots require only one antibody to a given protein, whereas sandwich immunoassays require two. Previous work has shown that high-affinity antibodies that react with a given HCP can be more sensitive than silver or fluorescent dye staining of gels.

1-D Western blots of final product provide a simple reliable orthogonal method for demonstrating product purity. Although the same antibody preparation may be used in both the immunoassay and Western blot, for the reasons noted above, the blot may provide additional information on trace proteins (and miss others), hence its orthogonal nature. *Table 6* presents the advantages and disadvantages of 1-D and 2-D Western blot methods for these purposes.

Below is a list of the major considerations for optimization of SDS-PAGE/Western methods:

- 2-D-gel format and size: The larger formats (18-cm length or longer) resolve proteins better and come in a variety of isoelectric focusing (IEF) application modes and molecular weight dimensions (e.g., gradients). Total protein staining of various formats can be studied to optimize the separation in both dimensions and thereby find the balance that separates most (or the maximum number) of HCPs.
- The load on 2-D gels should be optimized to balance sensitivity needed (higher load) versus resolution desired (lower load). The protein load, ionic content of the sample, and speed of separation need to be balanced to avoid over-heating and to optimize resolution. The stains or substrates to be used will influence this choice. Typically, more is loaded for the immunoblot (e.g., 200 µg) than for the total protein-stained gel or blot (e.g., 50 µg for silver stains). The difference in load may confound the separation to some extent; therefore, finding the right balance will require experimentation.
- Signal development for both the total protein (gel or blot) and the Western blot requires significant experimentation (e.g., dye types, exposure times, buffer conditions) to optimize. For example, because it is important to detect as many bands as possible, sometimes the signal is amplified so much that the most abundant species obscure the others, or the background increases and contrast is lost. A related issue is standardization and calibration of the densitometer or imager, which must be fine-tuned to achieve consistency under experimentally determined conditions. Both reagent and instrument optimization can require significant time to reproducibly establish the optimized conditions where signal is maximized and background is minimized (see also 〈1104〉).

- The transfer and blocking reagents require experimentation to identify the best conditions (such as buffers, instrument power/time, temperature, and blockers).

Table 6. Analytical and Characterization Western Blot Methods for HCP Testing

Method	Use	Advantages	Disadvantages	Approximate Sensitivity and Ability to Quantify HCPs
1-D gel, Western blot	Screening a large number of samples for consistency and presence of unknown protein bands that react with anti-HCP antibodies. May be used as part of GMP control system.	High-resolution separation; provides approximate MW information; relatively simple to run. Side-by-side analyses of samples provide powerful comparisons of lots.	Requires high-quality anti-HCP antibody source. Large excess of product in the gel can lead to nonspecific staining of product bands. SDS-induced denaturation of proteins leads to loss of conformational epitopes.	Not quantitative for HCPs unless a specific protein standard is used. Sensitivity depends entirely on the quality of the antibody.
Large-format 2-D gel (e.g., 20 × 25 cm) Western Blot with chemiluminescence or similar high-sensitivity detection.	Screening a small number of samples for consistency and presence of unknown protein spots reacting with HCP antibodies. Not suitable for routine use but may be used for characterization of specific lots of product or characterization of antibody reagents.	High-resolution separation of trace HCP impurities from product. Provides approximate MW and pI information on protein spots.	Requires high-quality anti-HCP antibody source. Large excess of product protein may obscure HCP spots. SDS-denaturation of proteins leads to loss of conformational epitopes. Produces highly technique-dependent results requiring skilled staff and sophisticated equipment.	Not quantitative for HCPs unless a specific protein standard is used. Sensitivity depends entirely on the quality of the antibody, but 100 ng/mg is possible.

5.3 Considerations for Chromatographic and Proteomic Methods

Mass spectrometric techniques for the detection, identification, and quantitation of individual HCPs are rapidly evolving and leverage much of the technology developed for proteomics. Furthermore, as new genomic databases become available (e.g., the CHO genome), mass spectrometric approaches are more useful. These methods (see also *Applications of Mass Spectrometry* 〈1736〉 for additional information) often combine sample preparation such as reduction, alkylation, and proteolytic digestion, followed by separation (e.g., with reversed-phase chromatography RP-HPLC) before introduction into a mass spectrometer that fragments all proteins thus providing an amino acid sequence for each peptide. The resulting sequence information is compared to the product sequence to identify product-related fragments and to a database related to the host (e.g., *E. coli*, CHO) to identify HCPs.

A challenge for MS analysis stems from the overwhelming number of product-derived peptides relative to impurity peptides. One approach to address this issue of competitive ionization of the peptides (also called ion suppression of the HCP peptides by the product peptides), is to apply LC-MS/MS analysis on partially resolved HCP preparations (e.g., HPLC fractions). This purification reduces the product contribution to total ions in the mass spectrometer. Other approaches are in development. Recent technological improvements such as 1) chromatography resins able to resolve effectively using MS-friendly mobile phases, 2) improved interfaces to front end LC and/or IEF separation systems, and 3) mass spectrometers with higher mass resolution, accuracy, and faster scan rates, now make it possible to identify and quantify specific HCPs in DS with a high degree of confidence. Major challenges in terms of sensitivity and quantitation of sufficiently large sets of heterogeneous HCPs, cost, and QC-related issues remain to be met before this technology can replace immunoassays for the control of HCPs in DS.

As a characterization method orthogonal to the HCP immunoassay, LC-MS/MS data may be used in two ways. First, if the MS-based method does not find HCPs in samples that were also below QL in the immunoassay, then these orthogonal techniques can increase confidence that the HCP ELISA did not miss an HCP that has co-purified. The detection limit for many LC-MS/MS methods is currently in the range of about 10 to 100 ng of HCP per mg of product. It is therefore sufficiently sensitive to rule out a single HCP being present at a high level and is more sensitive than gels. Second, if an impurity is identified at a high level or one that is a safety concern, then one can assess whether the purification process needs to be improved to remove it, whether the current control system is adequate, and whether development of a specific assay is required. Absence of signal does not prove absence of HCPs, but once an HCP is identified, the sensitivity of LC-MS/MS can be enhanced. LC-MS/MS is complementary to other HCP technologies and is increasingly valuable as an orthogonal method. Detailed discussion of all the proteomic approaches to the analysis of HCPs is beyond the scope of this chapter, in part because the field is evolving so rapidly that any review would become out of date quickly.

Intact HCPs also may be separated using HPLC with UV detection. The profile has been used for comparing different lots of null cell runs, for example. Readers are referred to *Chromatography* 〈621〉 for the core science behind HPLC chromatographic methods. Because HCPs differ in abundance, and the chromatography may not provide unique resolution of all proteins, an individual HCP must constitute about 1% (or less with good resolution) of the total protein population for possible detection (even then, a given HCP may be obscured by product, depending on its retention time). Nonetheless, investigation of unknown peaks as a general practice and comparisons of the chromatographic profiles of null cell run HCPs may be useful in determining the similarity of HCPs produced under different culture conditions.

As with the electrophoretic methods discussed in this chapter, reversed-phase separation of proteolytically cleaved product samples with UV detection may already be included in a GMP control system as part of product testing. The presence of new or unexpected peaks in the UV chromatogram may indicate the presence of an HCP impurity, although this method is not overly sensitive (~1% impurity if not obscured by other peaks). However, because no new testing or sample preparation is required, this opportunity to use an orthogonal method for determining purity should be considered.

Table 7. Analytical and Characterization Chromatographic and Proteomic Methods for HCP Testing

Method	Use	Advantages	Disadvantages	Approximate Sensitivity and Ability to Quantify
Mass Spectrometry – HPLC/electrospray ionization (ESI)-MS/MS or cIEF/ESI-MS/MS or MALDI-TOF of protein digests	Identification of individual HCPs. May be applied to the characterization of a purified product lot, or null cell run HCP standards.	Allows identification and monitoring of individual HCPs. May be combined with gels to identify the individual proteins seen in gels.	Low-throughput method requiring significant sample preparation, skilled staff, and sophisticated instrumentation.	100 ng/mg and semi-quantitative if used in the discovery mode for unknown HCPs. 10 ng/mg (or better may be possible) and quantitative if used with an analytical standard for a known HCP.
RP-HPLC/UV or RP-UHPLC/UV. May be applied to intact proteins or proteolytic digests	Suitable for comparing null cell run standards if proteins are well separated. Larger proteins may need limited proteolysis or reduction to improve resolution.	High resolution, separates HCPs based on hydrophobicity and size; direct comparison of null strain and product strain is possible via overlays.	Difficult to find a stationary phase and mobile phase combination able to resolve and elute all HCPs. Co-elution may hinder quantitation of specific proteins. High concentration of product peak(s) may interfere with detection of trace HCPs.	10,000 ng/mg (1%). Semi-quantitative unless used with an analytical standard for a known HCP.

5.4 Concluding Remarks on Supporting Technologies for HCPs

A number of various hybrid analytical techniques have been attempted over the years. One example is fractionation of HCP standards by ion exchange, followed by RP chromatography, and then an immunoassay or MS analysis (2-D MS) on the proteins in the final fractions. Alternatively, "product subtraction" has been evaluated to remove the bulk of the total protein from final product samples before gel electrophoresis of LC-MS/MS analysis. These hybrid methods for coverage determination are very labor intensive, and they may introduce artifacts or biases of their own (e.g., antibodies to product do not typically remove all the myriad of heterogeneous forms). As such, they have not found widespread application, although in individual cases they may provide insight into the nature of product impurities.

Electrophoresis, Western blotting, and immunoassay remain the most widely used methods for HCP reagent characterization and for demonstration of HCP clearance by purification processes, because they are robust, sensitive, and can cover a broad range of protein impurities. Immunoassay and (increasingly) mass spectrometry are highly complementary and the most powerful methods for monitoring residual HCP levels in samples and confirming their absence in final DSs.

6. USE OF HCP IMMUNOASSAYS FOR PROCESS DEVELOPMENT, CHARACTERIZATION, AND VALIDATION

As described previously, although HCP testing has limitations as quantitative assays, if the immunoassay can rapidly and robustly detect most of the HCPs, then it is extremely valuable during process development, characterization, and validation. HCP clearance at each intermediate process step under a variety of conditions is valuable data. Application to process validation requires that the assay be demonstrated to be scientifically sound, suitable for its intended purpose, and appropriately documented. As stated earlier, process pools can differ widely in terms of buffer components, product concentration, and HCP composition. At a minimum, acceptable spike recovery of the HCP standard into in-process pool samples is necessary to qualify test dilutions of the various pools in the assay. In addition, testing each sample type at multiple dilutions is helpful during development to verify that: 1) results are within the range of the assay, and 2) nonlinearity in sample dilution is considered. In cases where the process is shown to clear HCPs robustly, process validation may also be used to justify not having a routine HCP test as part of the cGMP control system.

Figure 5 illustrates data gathered during process development and validation and shows the dilution dependence for a number of in-process pools (*Pool 1* is after the first CCF purification step, *Pool 2* the next, and so on) through the process. *Figure 5* shows HCP ratio (in ng/mg) on the *y*-axis plotted against the product dilution in the test well on the *x*-axis. Each sample is serially diluted through three or four twofold dilutions. The starting dilution is determined by both the need to dilute samples with high levels of HCP into the range of the assay and the need to test only at dilutions where acceptable spike recovery has already been demonstrated. In *Figure 5*, the CCF has slightly more than 1 million ng of HCP per mg of product, meaning that a little less than one-half of the total protein in the CCF is product. The first column pool reduces the HCP level to just over 10,000 ng/mg. The second column has minimal HCP clearance; the third column reduces the HCP ratio to 1,000 ng/mg. The

final column in the process reduces the HCP level by an additional 2 logs, resulting in a final HCP ratio of 10 ng/mg. All of the intermediate pools show HCP ratio values that are independent of the dilution of the sample.

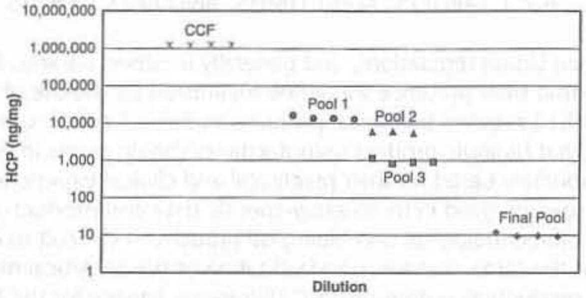


Figure 5. Example of HCP clearance by each purification step. Within a sample set, the first value is the most dilute in its dilution series.

Figure 6 presents a set of data for a different recovery process. As in *Figure 5*, the HCP ratio data for a serial twofold dilution series of each in-process pool sample are plotted. For CCF and the column 1 and column 2 pools, the HCP ratio (ng/mg) is independent of sample dilution (dilution linearity is achieved as in *Figure 5*). But after processing over the third column, distinct dilution dependence is apparent in the assay. This nonlinear dilution is likely associated with antigen excess relative to available antibody for the particular set of HCP(s) in the sample (i.e., a single or limited number of HCP species co-purify with the product). With product purification, more product must be put in the assay well for residual HCPs to be detectable in immunoassay. The product concentration in the assay may be increased with each purification step, and thus there is a similar increase in the concentration of the co-purifying HCP impurity. By column 3, the amount of the impurity likely exceeds the capacity of the available antibodies. This phenomenon is repeated in the final pool, which involves no HCP clearance but only buffer exchange by UF/DF. The reported value for these pools is the one obtained by diluting the sample to near the QL of the assay (the most dilute sample in the series) using a method described in *Table 4*, which is about 300 ng/mg in this case. Additional discussion of assay nonlinearity is found in the *4.3 Sample Linearity* subsection of *4. HCP Immunoassay Method Validation*.

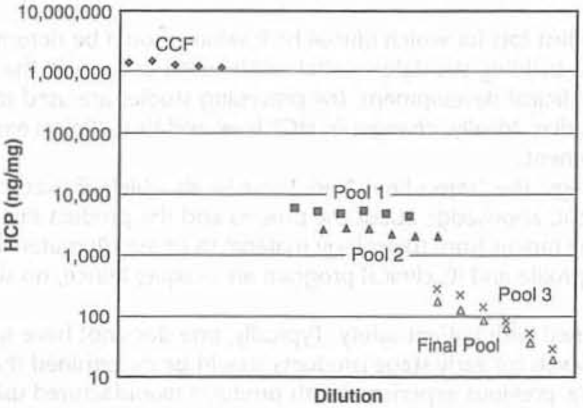


Figure 6. Example of HCP clearance with a purification process. Within a sample set, the first value is the most dilute in its dilution series.

6.1 Assays for Individual HCPs

In some cases, a manufacturing process might result in production of relatively high levels of a single HCP that can result in a bias in the reported results. As discussed above, in this situation the concentration of antibodies present in the total HCP assay may not be sufficient to capture all of that particular HCP present. In such cases, where a single, known HCP is present, another assay for that single HCP may be needed. For example, in the case of *E. coli* production cultures, co-expressing chaperones or other processing enzymes that aid in folding or disulfide formation can be produced in very high levels. In addition, these chaperones may be present in the immunogen used to develop the HCP assay reagents, in a conformation that is different from the way the protein is presented in samples. Thus, antibodies produced to chaperones not bound to product may not recognize, or may poorly recognize, product-bound HCPs. In this case, separate assays using appropriate antibodies may need to be developed. The existence of high levels of a single HCP is not unique to bacterial cell cultures and is possible with any cellular expression system. Another example is when a protein is intentionally added as part of the processing, then it is treated as a single HCP, and a specific assay will provide better data (than the total HCP immunoassay).

General Chapters

6.2 Control Strategy

6.2.1 TARGETS, ALERT LIMITS, AND REJECT LIMITS

In both United States and European Union regulations, and generally in other markets, HCPs are described as process-related impurities, and guidances state that their presence should be minimized by the use of appropriate, well-controlled manufacturing processes. U.S. 21 CFR 610.13 requires biological products to be "...free of extraneous material except that which is unavoidable...". ICH Q6B indicates that biologic product manufacturers should assess impurities that may be present and develop acceptance criteria for the impurities based on their preclinical and clinical experience. However, regulatory guidance also recognizes the inherent challenges involved in using assay-specific data and product- and process-specific reagents. Guidance states "The absolute purity of biotechnological and biological products is difficult to determine and the results are method-dependent" (ICH Q6B), and, "In the same manner, standardization of the analytical methods would be problematic since the reagents used are product and production system-related" [European Agency for the Evaluation of Medicinal Products (EMEA) 1997].

Although FDA guidance describes the need to test for HCPs, it also allows, through validation, removal of the release testing requirement, once clearance has been shown to be consistent. An expectation exists that, whenever possible, impurities that were introduced by manufacturing processes and remain in products should be below detectable levels based on a highly sensitive analytical method. However, an appropriately conducted clearance study performed as part of process validation can be an acceptable substitute for lot-to-lot (C of A) testing (FDA 1997), even when low levels of HCPs are present in the DS. In addition, "If impurities are qualitatively and quantitatively (i.e., relative amounts and/or concentrations) the same as in the DS, testing of the DP is not necessary" (ICH Q6B). This is the case for HCPs, the concentration of which in the DP may be inferred from the DS, because the manufacturing process does not introduce, or clear, HCPs when the DS is converted to the DP. Thus, in these instances, HCP testing of the DP is generally not required.

The overarching goal is to develop processes that minimize the quantities of residual HCPs in DSs. When using HCP immunoassays as part of a manufacturing control system, limits for acceptable results need to be established. The "target" for HCP in final product gives the purification development group an objective for the process, which may or may not be achievable. Antibody processes with highly selective affinity chromatography steps (e.g., using Protein A) are sometimes able to achieve a sensitivity of 1 ng of HCP per mg of protein. Target HCP levels determine the design criteria that are then used to develop the process.

Preclinical toxicology lots are the first lots for which official HCP values should be determined, because at this point in product development, the investigator is building the data set that will be used to support the safety of subsequent clinical studies. As the molecule advances through clinical development, the preceding studies are used to support the projected safety (and risk) associated with subsequent studies. Ideally, changes in HCP level and thus clinical exposure should decrease as the product moves through clinical development.

As part of an overall control strategy, the "reject limits" are those levels which, if exceeded, render the product no longer fit for use. During product development, knowledge about the process and the product increases; therefore, it is expected that reject limits can be tightened as one moves from toxicology material to phase I/II material, to phase III, and finally to commercial material. Each drug candidate profile and its clinical program are unique; hence, no single numerical limit is applicable to all.

Reject limits are primarily concerned with patient safety. Typically, one does not have specific data documenting "unsafe" levels of HCPs. Rather, acceptable levels for early stage products should be determined through a risk assessment (including non-clinical data, available literature, previous experience with products manufactured using the same or a similar cell line, etc.). For commercial products, the acceptable levels are also based on the experience gathered during clinical trials. Because the HCP impurity profiles may be influenced by the purification process, the registration batches used in the pivotal clinical trials must be representative of the to-be-marketed process. Reject limits for commercial product should be set to exclude HCP values that are outside the range of clinical experience upon which the risk/benefit decision was made for the biopharmaceutical.

Alert limits, trend limits, or action limits (different companies use different terms) reflect considerations of manufacturing consistency. In a commercial process, there should be sufficient manufacturing history to be able to define out-of-trend results. However, early in product development, where there is little or no manufacturing history, action limits may be used to set upper limits on the expected HCPs, based on experience with similar products for which there is a history, or in comparison to the target value. Exceeding these limits should trigger an investigation to determine whether the lot is releasable. Orthogonal measures of product purity are valuable in such investigations.

With the emergence of orthogonal measures of purity, any non-product related atypical signals should be evaluated. Efforts should be made to revise the purification process to remove any unwanted HCPs present at higher than desirable levels (based on, for example, clinical and non-clinical data, available literature, etc.), or a specific justification of its acceptability must be provided. This is accomplished as part of a risk analysis that considers the clinical exposure similar to that described above for immunoassays.

In cases where HCP assays are improved or changed due to unforeseen events, the reject limits may need to be modified, even increased in some cases (if the new assay has broader coverage). However, it is essential that samples be tested in bridging studies to ensure that no changes in quality would be missed in the new, improved assay. In some cases, improved assays

give higher values, so bridging studies (head-to-head with original versus improved assay) will be needed to justify the new specification limit.

6.2.2 SAFETY CONSIDERATIONS IN SETTING LIMITS

HCP specification limits in units of ng/mg are typically set using process capabilities and also on the basis of clinical experience (i.e., the levels used in trials where the clinical outcomes are known). Although there are several theoretical safety concerns about HCP impurities, the immunogenicity of the impurity is a primary consideration. An immune response may include formation of antibodies directed against the HCP or directed against the therapeutic protein, where the HCP is believed to have acted as an adjuvant. Readers are referred to ⟨1106⟩ for a more in-depth discussion of factors influencing immunogenicity. When patients receive more than one biotherapeutic protein with their associated impurities, immunogenicity is an even greater concern. Although high-dose products may contain a higher mass of residual HCPs per dose and this should be considered during risk assessments, the potential effect is not necessarily predictable of a clinical outcome. In some cases, very low levels of certain HCPs have been shown to have clinical effects, whereas high levels of other HCPs have had none.

Different limits may be set, depending on whether the product has only one or a very limited number of co-purifying HCPs or it has a multiplicity of HCPs, each present as a small fraction of the total HCP level in the product. This could lead to a separate assay and specification for individual HCP(s) that cannot be cleared from the process and are expected to be present in the DS and may have bioactivity.

The host cell origin can also be a consideration. It is possible that HCPs from human cell lines (e.g., HEK293 cells) may pose less immunogenicity risk than HCPs from more distantly related species because of sequence homology. However, if these proteins are normally intracellular, then they might still be immunogenic and seen as "foreign" by the patient's immune system. If such antibodies develop, then they could cross-react with endogenous human proteins and neutralize or render ineffective one or more necessary biological systems. As a consequence, arguments can be made on both sides; hence, the risks based on production cell line (microbial versus mammalian) are generally believed to be the same and cannot be practically informative *a priori*.

In addition to considering immunogenicity, the HCP may be bioactive and a risk. For example, a hamster homolog of a therapeutic protein expressed in CHO cells could be sufficiently similar to the product to co-purify. Alternatively, an anti-human cytokine antibody product expressed in CHO cells could bind to the homologous hamster cytokine, and the complex could co-purify with the antibody product. In both cases, the homologous hamster proteins could be biologically active when administered to patients.

7. SUMMARY AND CONCLUSIONS

Biopharmaceutical products should be as free of residual HCPs as reasonably possible. The industry standard for measuring HCPs is a sandwich immunoassay using polyclonal antibodies directed against the broadest possible spectrum of potential impurity proteins. Because the diversity and proportion of HCPs in the calibration standard is never matched exactly to the HCPs in any sample and due to the broad range of antibodies in the immunochemical reagents, different HCP assays will quantify individual HCPs to a differing extent. Thus, the single numerical value readout of the HCP immunoassay is "immunologically weighted". Highly immunogenic proteins will overquantify relative to the average population, and weakly immunogenic proteins will underquantify. Some proteins may not induce an immune response in the animal species used to generate antibodies, and the HCP immunoassay will be blind to those proteins. Because of this possibility, orthogonal methods for judging product purity also should be included in the control system. The best strategy is to use the HCP immunoassay in conjunction with other analytical tools.

Finally, one should use caution in extrapolating safe HCP values across products. No single specification limit will suffice. Differences in the biological properties of particular HCPs that co-purify with product, the route of administration, the patient population, and product/HCP dose all contribute to possible factors influencing patient risk. Over the history of recombinant biologic therapeutics, there have been examples where individual co-purifying proteins have been immunogenic, acted as adjuvants to increase the patient immune response to the therapeutic protein, or had biological activities themselves. Although these examples exist, they are rare, and overall there is an excellent safety record for biologics. In spite of the limitations and complexities of HCP assays, careful and diligent development and application of these assays have helped to ensure patient safety for millions of doses. HCP analysis continues to be a complex problem demanding time, resources, and careful thought by biopharmaceutical manufacturers with the goal of ensuring that products consistently meet the standard of having as low an HCP impurity level as can reasonably be achieved.

8. BIBLIOGRAPHY

EMEA. 1997. CPMP position statement on DNA and host cell proteins (HCP) impurities, routine testing versus validation studies. London: European Medicines Agency. CPMP/BWP/382/97.

FDA. 1997. Points to consider in the manufacture and testing of monoclonal antibody products for human use. www.fda.gov/CBER/gdlns/ ptc_mab.pdf. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsforManufacturers/UCM153182.pdf. Accessed 2013-11-01.
ICH. 1999. Guidance for Industry Q6B Specifications: Test procedures and acceptance criteria for biotechnological/biological products. http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html. Accessed 2013-11-01.
ICH. 2005. Validation of analytical procedures: Text and methodologies. Q2(R1). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. Accessed 2014-02-26.

〈1136〉 PACKAGING AND REPACKAGING—SINGLE-UNIT CONTAINERS

SCOPE

This chapter provides guidance for the packaging and repackaging of single-unit containers, and for the use and application of unit-of-use packaging. Although the chapter is intended for use by drug manufacturers, repackagers, and pharmacists, the information in the chapter may also be useful for suppliers of packages and packaging components. For the definition of specific types of packaging, see *Packaging and Storage Requirements* 〈659〉.

SINGLE-UNIT CONTAINER

Single-unit containers that package a prescription drug to be dispensed directly to the patient are required to be child-resistant. Single-unit packaging intended for institutional or hospital use may or may not be required to be child-resistant. Single-unit containers that are child-resistant include supported blisters, such as separate, peel, push, and tear notch, and enclosed or in-card blisters, such as pull tabs and slide packs.

PACKAGING MATERIALS

Materials used to manufacture single-unit packaging containers include glass and plastic. Glass used as a primary packaging component should meet the requirements of *Containers—Glass* 〈660〉. Plastic materials as a primary packaging component should meet the requirements of *Plastic Packaging Systems and Their Materials of Construction* 〈661〉, *Plastic Materials of Construction* 〈661.1〉, and *Plastic Packaging Systems for Pharmaceutical Use* 〈661.2〉. The test for moisture permeability may be carried out as described in general test chapter *Containers—Performance Testing* 〈671〉.

PACKAGING CLOSURE TYPES

Reclosables and nonreclosables may be used for solid, semisolid, and liquid dosage forms. Both must be packaged in compliance with the 16 CFR 1700.15 standards. The Poison Prevention Packaging Act (PPPA) of 1970 requires in certain cases the use of special packaging—child-resistant and senior-friendly. Child-resistant packaging protects children from serious injury or illness resulting from ingesting or handling hazardous products including drugs.

Because drugs packaged in unit-of-use packaging are intended to be dispensed to the consumer without repackaging by the pharmacist, the manufacturer or repackager is responsible for the special packaging of PPPA-regulated substances in unit-of-use containers (16 CFR 1701.1).

Reclosables

Reclosables are containers with suitable closures that may incorporate tamper evidence and child-resistance capabilities. Reclosables may be used for glass or plastic containers.

Nonreclosables

Nonreclosables are containers with closures that are nonreclosable, such as blisters, sachets, strips, and other single-unit containers. Nonreclosables may include packs such as cold-formed foil blisters, foil strip packs, and PVC/Aclar® combining multilayer materials that are thermo-formed or cold-formed foil blisters. Nonreclosables may be child resistant depending on the intended use and place of use. Household nonreclosables are subject to the PPPA as defined in 16 CFR 1700.14. However, because of some unit-dose designs, not all unit-dose packages comply with the PPPA.

UNIT-OF-USE

Unit-of-use packaging, when provided by the manufacturer, offers some of the following attractive advantages. (1) A dosage form can be dispensed to a patient in the manufacturer's original container, a practice that recognizes that the suitability of the container has been established on the basis of the manufacturer's stability studies. (2) The counting and repackaging of dosage units in the pharmacy is eliminated, thereby reducing the possibility of human error. (3) The pharmacist is able to affix the label for the patient onto the unit-of-use package and is free to use the manufacturer's expiration date as the beyond-use date. (4) The number of dosage units in a single unit-of-use package may be determined on a case-by-case basis. (5) Patient compliance is improved. (6) The unit-of use package can protect against counterfeiting because traceability of product is ensured through bar coding techniques and National Drug Code (NDC) numbers.

Unit-of-use packaging, when provided by repackagers, offers the same attractive advantages as those offered by the manufacturer. However, unit-of-use repackagers should conform to all requirements as presented in *Good Repackaging Practices* ⟨1178⟩. There are a number of reasons why repackagers produce unit-of-use packaging, for example, (1) requests from institutions, (2) better inventory control, (3) reduced dispensing times, and (4) variations in some drug therapies.

The packaging of a unit-of-use system may be a multiple-unit or single-unit container. A unit-of-use system may contain a drug product in a liquid, semisolid, or solid dosage form (see also *FDA Guidance for Industry, Container Closure Systems for Packaging Human Drugs and Biologics*). [NOTE—The terms "unit-of-use package" and "unit-of-use container" may be used interchangeably.]

Unit-of-Use Labeling

The unit-of-use containers are labeled to include expiration dates, the manufacturer's lot number, the NDC designation, and bar codes as provided in the *Labeling* section of *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling* and in *Good Repackaging Practices* ⟨1178⟩. Some of the advantages of having bar codes on the label include reduced medication errors, improved inventory control, and improved access to medication identity. The labeling covers information placed in the container by the manufacturer (see *General Notices and Requirements*). Acceptable labeling can range from full labeling, such as that for multiple-unit containers, to abbreviated labeling when the container is too small to include all of the text. Full labeling may also be provided on the carton if it is not present on the immediate container.

Unit-of-Use—Repackaging and Reprocessing

Unit-of-use containers are reprocessed or repackaged as instructed by the manufacturer. A unit-of-use package that is a blister package may not be reprocessed by a pharmacist once it has been deblistered from a unit-dose container (see *General Notices and Requirements* for application of the appropriate beyond-use date for a multiple-unit or unit-dose container). Deblistering is the process of removing medication from a blister-type container. However, under current Good Manufacturing Practices (cGMPs) and tight quality controls, the manufacturer or contract repackager may repackage and reprocess unit-of-use containers.

Information from Manufacturers

The manufacturer should provide appropriate stability information that can be used to determine appropriate labeling, storage, and shipping statements that will properly inform patients and practitioners. The manufacturer may make other assurances based on product information on packaging and distribution arrangements. In the event that a product is not to be repackaged, the manufacturer may so state in the labeling. The manufacturer also includes labeling and information necessary for optimal handling by the practitioner and the patient. The labeling and information should be bar coded to eliminate medication error and promote medication traceability.

Responsibility of the Dispenser—Labeling

The labeling on a unit-of-use container also includes a label added at the dispensing stage by the pharmacist. Prior to dispensing the unit-of-use package, the dispenser shall add label(s) that provide the following information:

1. The name of the patient;
2. The name and strength of the drug product, the directions for use as prescribed by a doctor or health-care provider, and the name of the prescriber; and
3. Any storage instruction, beyond-use date, and other information as deemed appropriate by federal and state laws.

In the pharmacy setting, pharmacists are encouraged to use bar codes, in conjunction with computerized prescription orders, to confirm that the right drug is being dispensed to the right patient. Bar coding would minimize errors and create an opportunity for medication traceability and accountability.

Quality Control of Packaging System

The packaging system shall meet the general considerations for system suitability, protection, safety, and performance characteristics as described in *FDA Guidance for Industry, Container Closure Systems for Packaging Human Drugs and Biologics*, and in *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and *Containers—Performance Testing* ⟨671⟩.

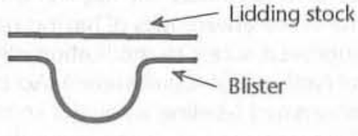
REPACKAGING A SINGLE SOLID ORAL DRUG PRODUCT INTO A UNIT-DOSE CONTAINER

Repackaging of solid oral drug products, such as tablets and capsules, into unit-dose configurations is common practice both for the pharmacy that is dispensing drugs pursuant to a prescription and for the pharmaceutical repackaging firm. The following section contains minimum standards to be used as a guideline for repackaging practices.

Repackaging preparations into unit-dose configurations is an important aspect of pharmaceutical care and of optimization of patient compliance. For purposes of this chapter, there are two types of repackaging: the first involves pharmacies that dispense prescription drugs, and the second concerns commercial pharmaceutical repackaging firms.

Materials

Blister packages offer a wide array of designs both in functionality and in appearance. Various packaging materials are used to create blisters that are tailored to provide optimum performance. The blister container consists of two components: the blister, which is the formed cavity that holds the product, and the lid stock, which is the material that seals to the blister, as shown below.



Schematic Presentation of a Typical Blister Pack

Because of the variety of blister films available, film selection should be based upon the degree of protection required. The choice of lid stock depends on how the blister is to be used, but generally the lid stock is made of aluminum foil. The material used to form the cavity is typically a plastic, which can be designed to protect the dosage form from moisture. There are widely varying degrees of moisture protection now available. For purposes of this general chapter, they are referred to as nominal, medium, high, and extreme moisture barrier properties.

POLYVINYL CHLORIDE

The most commonly used blister material is polyvinyl chloride (PVC). This material, which provides a nominal or zero barrier to moisture, is used when the product does not require effective moisture protection. PVC is available in a range of gauges and can be made opaque or can be tinted with pigments to block out specific light wavelengths.

The thickness of the PVC used is determined by the depth and size of the cavity to be formed. Because the plastic thins during the blister-forming process, care should be taken to ensure that the finished blister provides sufficient protection from light (if required) and that it is strong enough to adequately protect the dosage form. Common gauges of PVC used in the pharmaceutical industry range from 7.5 to 15 mil (0.0075 to 0.015 inch).

BARRIER FILMS

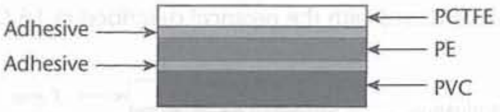
Many drug preparations are extremely sensitive to moisture and therefore require high barrier films. Several materials may be used to provide moisture protection. Barrier films commonly used in the pharmaceutical industry are described below.

PVC/PCTFE laminations: Polychlorotrifluoroethylene (PCTFE) film[1] is a thermoplastic film made from polychlorotrifluoroethylene fluoropolymer. The PCTFE film is laminated to the PVC by an adhesive layer between the PVC and the PCTFE film (duplex structure),

Duplex Structure

or by a layer of polyethylene (PE) between the PVC–adhesive and the PCTFE–adhesive layers (triplex structure).

[1] PCTFE film is available from Allied Signal (as Aclar®) and from other sources.



Triplex Structure

By using various gauges of PCTFE film, medium to extreme moisture barriers can be obtained.

PVC/PVdC laminations: PVC/PVdC is a film in which the PVC is coated with an emulsion of polyvinylidene chloride (PVdC), as shown in the duplex structure pictured below.

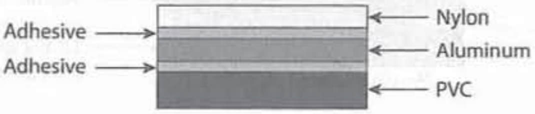
Duplex Structure

The PVdC layer is specified in g/m² and can be constructed to provide medium to high barrier protection. The coating weights commonly used in the pharmaceutical industry are 40, 60, and 90 g/m², and the film is offered with or without a middle layer of polyethylene (PE), as shown in the triplex structure below. The polyethylene is used with heavier coating weights, such as 60 and 90 g/m², to improve the thermoforming characteristics of the blister cavity.

Triplex Structure

Polypropylene: Because of its morphology, polypropylene (PP) serves as a good moisture barrier, its spherulitic structure creating an arduous path for water molecules to traverse. Although not commonly used as a pharmaceutical blister film in the United States, PP provides an economical alternative to medium barrier materials and is used in Europe as an alternative to PVC.

Cold form foil: This material is used for products that are extremely hygroscopic or light sensitive. It is an extreme moisture barrier and consists of three layers: PVC, aluminum foil, and nylon.



Cold Form Foil

LID STOCK

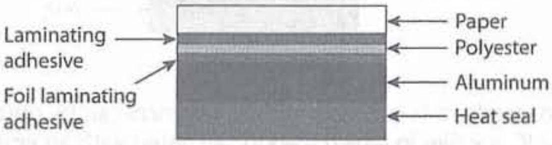
The lid stock is sealed to the molded blister as described above. Different designs of lid stocks are available, and selection of a particular design depends on how the package will be used. Standard designs—peelable, child-resistant peelable, and push-through—are described below. The primary component of lid stock is typically aluminum, and its gauge varies from 18–25 µm (0.0078–0.001 inch). The side of the aluminum foil laminate in contact with the product provides the heat-sealable layer that forms the seal to the blister material. The heat-seal coating should be capable of forming an adequate seal with the blister film to which it is intended to seal. The materials used in the makeup of the heat-seal layer meet the requirements of 21 CFR 175 and 177.

Peelable: Peelable foil, commonly used in an institutional setting, consists of several layers, as shown below, and can be peeled away from the blister. [NOTE—For child-resistant peelable foil, a layer of polyester with the appropriate adhesives would be added.] With the peelable-foil lid stock, which is used in conjunction with blister tooling, a three-step process is required to open the blister.

Peelable Foil Construction

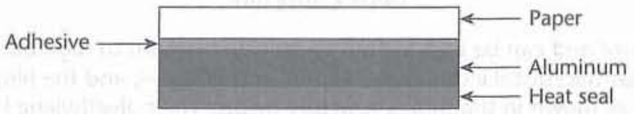
First, the blister cavity must be separated from the rest of the blister card. Next, the paper and polyester layers are pulled back from an unsealed area. Finally, the product is pushed through the remaining aluminum foil. It is important to note that use of this type of foil structure helps make the package more child resistant. However, if child-resistant packaging is required,

the package design should be tested in accordance with the protocol described in 16 CFR 1700, the Poison Prevention Packaging Act.

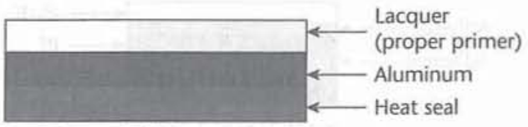


Child-Resistant Foil

Push-through: There are two commonly used types of push-through foil: one with a paper outer layer separated from the aluminum by a layer of adhesive, and one without paper. The paper outer layer serves as an aesthetic and makes it possible to print on the back of the blister.



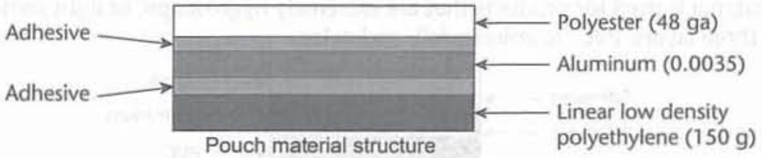
Push-through foil (with paper)



Push-through foil (no paper)

OTHER PACKAGE STYLES

Other types of packages used for unit-dose packaging of solid dosage forms are strip packs, pouches, and sachets.



Pouch material structure

PROCESS

Unit-dose packages can be formed and sealed in a variety of ways. Larger scale repackagers may use thermoformers that accomplish these functions in-line, while smaller repackagers may purchase preformed blister material. This section begins with an overview of the process involved in thermoforming a blister, the fundamental process that also applies to other unit-dose package types such as pouches. The overview is not intended to be all-encompassing, but it highlights the major operations along with their critical parameters.

Thermoforming a Blister Unit-Dose Package

The complete thermoforming process consists of four basic stations where the following operations occur: forming, filling, sealing, and finishing. Thermoforming requires the use of heat and air in forming the blister. The lid stock material is sealed to the blister cavity material for a defined time (the stroke of the machine) at the point where the heat plate closes on the two materials.

FORMING STATION

Prior to entering the forming station, the blister material passes through a heating unit where the blister material is heated uniformly in stages to ensure proper formation. Because different plastics have different softening points, careful attention must be paid to determining the proper temperature of the heating station, which often has multiple temperature zones. The temperature, based on the blister material used and on the speed at which that material travels through the heating station, is a critical parameter for optimal performance. At the forming station, the blister material is heated to the point where the plastic softens sufficiently to allow the cavity to be formed. The blister material is drawn from a reel-mounted roll (referred to as the web) and pulled through the machine. A splicing table is located at the reel unwind to provide room for a second roll of

blister material to be readily available for splicing and resumption of the packaging process. An unwind device may be installed to aid in moving the blister material from the roll as adjusted for a specific index.

Once the blister material is properly heated, compressed air is generally used to form the blister cavity. Upper and lower forming dies close on the blister material as air is introduced, forming a blister that corresponds to the size of the cavity. A plug assist may be necessary, depending on the material and size of the cavity. The plug assist ensures a uniform thinning of the blister material to optimize the protective characteristics of the formed material. Once the blister material is formed into the desired blister configuration, it is advanced to the filling station.

FILLING STATION

The product is loaded into the blister cavity at this station. An automated filling device may be used, or the cavities may be hand filled. The critical parameter at this station is proper filling of the formed blisters.

SEALING STATION

At this station, the lid stock is sealed to the filled blister cavity, using heat and pressure for a defined dwell time. The critical parameters to be considered at this station are temperature, pressure, and dwell time.

The lid stock material is staged on a roll above the blister cavity and may be preprinted or printed online. Lot numbers and expiration dates may be applied at this point. Preprinted lid stock materials will require a print registration system to control the position of the printing relative to the blister cavity. The critical parameters at this part of the station include legible and correct labeling.

FINISHING STATION

The finishing station encompasses all other steps in the packaging process, including embossing, perforation, and cutting. Embossing involves application of a lot number and expiration date to the package. Steel type is used to emboss information on the edges of the blister package. One of the critical parameters at this station is package integrity. It is important that the embossing, perforation, and cutting processes do not compromise the blister, lid, or seal. The quality of the embossing is another critical parameter in the process. The embossing must be legible and correct, and must include all required information.

Pouch Unit-Dose Packages

The pouch process is also a form, fill, and seal operation, but it does not provide a defined, formed cavity as does the thermoforming process. Although the equipment used to form pouch unit-dose packages may function differently from that described for thermoforming a blister, the main operations (form, fill, and seal) and critical parameters at those stations are quite similar. [NOTE—See the critical parameters defined in the section on thermoforming.]

The strip-pack process involves the drug product being dosed into a three-sided, formed pouch. Once filled with the drug, the machine seals the pouch, forming a strip of sealed unit-dose pouches. The basic flow of the process begins with the drug situated above the pouch material. One roll of strip-pack material is used to form the pouch. This is accomplished by moving the material over a device that forces the material to fold into two equal sides. The sides and bottom are sealed prior to dosing. The strip pack may be cut later during the equipment processing or roll continuously and be manually cut. Temperature and dwell time are the main critical factors for this equipment.

Preformed Unit-Dose Packages

Preformed containers are sealed either by heat or by adhesion. Heat sealers may be manual units requiring hand pressure application or automated units that provide a more controlled pressure for sealing.

Heat sealing may be accomplished through the use of manual tabletop equipment, which is generally operated at a set pressure. Critical parameters with these devices are pressure and temperature control because undesirable variation in these parameters may yield inadequate seals.

Critical Parameters

In order to ensure that the finished container performs as intended, qualification of critical parameters should be determined. Typically, validation of a packaging line consists of qualification of the installation, operation, and performance of a packaging system.

INSTALLATION QUALIFICATION

Equipment should be installed and found to be in proper working condition prior to use.

OPERATIONAL QUALIFICATION

Operational qualification should be performed to establish that the equipment operates within the manufacturer's specified ranges. Incoming utilities for the equipment, such as air, electricity, etc., should be monitored and checked periodically.

PERFORMANCE QUALIFICATION

Performance qualification should be done to establish that the equipment performs properly with the required materials to produce a container that functions as intended. The critical parameters include forming temperature and pressure, sealing temperature and pressure, and dwell time at the seal station. Qualified ranges should be readily available in a reference source for the setup of equipment. Re-evaluation may be necessary with changes to equipment, materials, or process.

In-Process Inspections

Strict controls covering the packaging and labeling processes should be in place. The final container should be evaluated for performance in each of the stations previously described. Specifically, the formed container should be inspected visually to ensure that it is properly formed. Evaluation of the filling station should include a check to ensure that the unit dose is properly filled (i.e., that the correct product is present). The sealing station should be evaluated to ensure that a proper seal has been made and that the moisture permeation specifications of the sealed container have been met. A visual examination of the package should be performed to ensure that the final steps of the packaging process are acceptable.

Repackagers and dispensers should use a standard inspection plan to verify the adequacy of the package. A visual inspection should be performed to verify that the correct product is in the proper packaging materials with correct labeling. Seal integrity should be evaluated, using vacuum testing,[2] helium testing, tear testing, and other testing methods suitable to establish whether seal integrity is maintained.

PERFORMANCE

The primary purpose of the unit-dose package used in the packaging of a drug preparation is to ensure that, until its intended expiration date, there is adequate protection from the environment as the dosage form is distributed and stored. It is also essential that the materials used do not interact with the dosage form.

When determining what type of package to use in the repackaging operation, consideration must be given to the dosage form's sensitivities (if any) to the storage and distribution environments (e.g., temperature, light, and moisture).

The properties of the finished container are defined by the materials used in constructing the unit-dose container, and by the process used to form and seal the container. As discussed in *Materials*, there is a wide variety of commercially available film structures that provide unit-dose containers with a range of moisture and light protection. Suppliers of these materials typically provide quantitative data, obtained from well-established test methods, to highlight the protective properties of their material. These data are based on flat sheets of the film, not on the formed container.

It is critical to understand that once the film is formed, protective properties change because the overall thickness of the film decreases as the blister cavity is formed. Usually the change is a decrease, especially in the case of barrier properties. However, the extent of change will vary with the type of film structure used and is also highly dependent on the container-forming process used (see *Process*). Further, a suboptimal seal on the formed container will decrease the protective properties of the container. Insufficient temperature, time, or pressure during a heat-seal operation may enable the passage of moisture or oxygen through the seal area over time, which may have an effect on the dosage form. In addition, if the seal area is designed with insufficient surface area, the same problem may occur. To ensure a good seal, a minimum sealing distance of 3 mm from the edge of the blister cavity to the nearest edge or perforation is recommended. Therefore, it is important to measure the performance of the formed and sealed container rather than the performance of the flat sheet.

Moisture is a critical factor in preparation integrity. *Containers—Performance Testing* ⟨671⟩ describes how to determine and classify moisture permeation rates. If the manufacturer's labeling includes "Protect From Moisture," the repackager shall utilize a high barrier film.

If light protection is required for a drug preparation, the repackager should follow the requirements for light transmission established under *Containers—Performance Testing* ⟨671⟩. Again, this testing should be conducted on the formed container, because the light-protective properties of the film are compromised once the film is thinned during the forming process. It is recommended that these tests, in conjunction with any guidance provided by the manufacturer, be considered appropriate for any container–closure system used in repackaging a drug preparation.

[2] Vacuum testing consists of placing samples from the packaging operation into a jar filled with water. A lid is placed over the samples to fully immerse them in the water. A container lid is applied to create a seal effective enough to create approximately 25 cm of vacuum. The vacuum pump is set, and the samples are tested for approximately 1 minute, removed from the water, wiped down, and opened to determine whether the inside of the unit-dose cavity or pouch is wet. This process should be adjusted until it is under control, and additional testing may be performed to ensure that the seal integrity is consistently acceptable. Wetness indicates a defective seal and therefore the potential for the drug to degrade when exposed to the atmosphere. Defective packages must be removed from further use.

BEYOND-USE-DATE

In the absence of stability data for the drug product in the repackaged container, the beyond-use dating period is one year or the time remaining until the expiration date, whichever is shorter. If current stability data are available for the drug product in the repackaged container, the length of time established by the stability study may be used to establish the beyond-use date, but must not exceed the manufacturer's expiration date.

As stated in the *General Notices and Requirements*, the dispenser must maintain the facility where the dosage forms are packaged and stored at a temperature such that the mean kinetic temperature is not greater than 25°. The plastic material used in packaging the dosage forms must afford better protection than polyvinyl chloride, which does not provide adequate protection against moisture permeation. Records must be kept of the temperature of the facility where the dosage forms are stored, and of the plastic materials used in packaging.

MINIMUM REQUIREMENTS

The previous sections serve as a general introduction to repackaging by providing a basic understanding of materials selection, the form-fill-seal process, and the importance of performance of the sealed container. In this section, certain minimum requirements for repackaging, which must be met, are described in more detail.

Personnel

Each person with responsibility for the repackaging of a preparation shall have the education, training, and experience, or any combination thereof, to perform assigned functions in a manner such that the safety, identity, strength, quality, purity, potency, and pharmaceutical elegance of the drug dosage form are retained. Training should be documented.

Personnel engaged in the repackaging of a preparation shall wear clean clothing appropriate for the duties or processes performed.

Facility

The repackaging facility may require areas of low relative humidity, and temperature conditions should meet controlled room temperature requirements specified in *Packaging and Storage Requirements* ⟨659⟩.

Equipment

Equipment used in the repackaging of a preparation shall be of appropriate design and suitably located to facilitate operations for its intended use. Its design should allow for cleaning to preclude cross-contamination as well as for maintenance to be performed. Equipment shall be constructed so that those surfaces that contact components or a preparation are not reactive, additive, or absorptive. Any substances required for operation, such as lubricants or coolants, shall not come into contact with components or a preparation.

Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination. Preventive maintenance should be performed at appropriate intervals in accordance with the equipment manufacturer's recommendation. Any instruments used to monitor critical parameters should be calibrated on a defined schedule.

Process

Steps should be taken to determine the critical process parameters (e.g., seal temperature, dwell time) in operating the equipment. Set points for these parameters should be documented and procedures established to ensure that they are adhered to each time the equipment is operated.

Labeling

The labeling requirements for a commercial repackager and a pharmacist are different. For example, the commercial repackager must comply with 21 CFR 201.1, but the pharmacist or dispenser does not have to comply with this requirement. If stability data are unavailable, the dispenser shall repackage only an amount of stock sufficient for a limited time and shall include product name and strength, lot number, manufacturer, and appropriate beyond-use date on the label. When quantities are repackaged in advance of immediate needs, each preparation must bear an identifying label, and the dispenser is required to maintain suitable repackaging records showing the name of the manufacturer, lot number, expiration date, date of repackaging, and designation of persons responsible for repackaging and for checking. The repackager or dispenser will use documented controls to prevent labeling errors.

Materials

The repackager or dispenser shall place an appropriate beyond-use date on the label and package in appropriate materials. Materials used by the repackager shall not be reactive, additive, or absorptive, and must meet the requirements described in 21 CFR 175 and 177.

Storage

The dispenser shall rotate and monitor stock closely to ensure that the dispensing of preparations is on a first-in-first-out (FIFO) basis. The repackager or dispenser shall store preparations under required environmental conditions (e.g., controlled room temperature with a mean kinetic temperature not higher than 25°).

Drug Product

The repackager or dispenser shall examine preparations for evidence of instability such as change in color or odor, and shall exercise professional judgment as to the acceptability of a package.

Complaints

The repackager or dispenser will maintain written procedures describing the handling of written and oral complaints regarding a drug product and will ensure that complaints are investigated and appropriately resolved.

Returned Goods

Policies and procedures relating to returned goods should be developed to ensure proper handling.

Reprocessing

Reprocessing of repackaged unit-dose containers (i.e., removing medication from one unit-dose container and placing it into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided the original beyond-use date is maintained, and provided the integrity of the blister is ensured.

Special Considerations

If a product is known to be oxygen sensitive or if it exhibits extreme moisture or light sensitivity (e.g., cold form foil), it shall not be repackaged. If a product is refrigerated, it shall not be repackaged unless proper environmental conditions and suitable materials are available. Certain drug products (such as oncologic agents, hormones, or penicillin derivatives) require special handling because they are considered very potent or toxic, and because transfer of any portion of these products to another product could have deleterious effects.

REPACKAGING NONSTERILE SOLID AND LIQUID DOSAGE FORMS INTO SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS

The following guidance is intended for those engaged in pharmaceutical dispensing, and does not apply to commercial dispensing. An official dosage form is required to bear on its label an expiration date assigned for the particular formulation and package of the article. This date limits the time during which the product may be dispensed or used. Because the expiration date stated on the original manufacturer's container–closure system has been determined for the drug in that particular system and is not intended to apply to a product that has been repackaged in a different container, repackaged drugs dispensed pursuant to a prescription are exempt from using the expiration date from the original manufacturer's package. However, under no circumstance should the repackaged pharmaceutical preparation's expiration date exceed the original manufacturer's expiration date. It is necessary, therefore, that other precautions be taken by the dispenser to preserve the strength, quality, and purity of drugs that are repackaged for ultimate distribution or sale to patients.

The following guidelines and requirements are applicable where official dosage forms are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

Labeling

It is the responsibility of the dispenser to place a suitable expiration date on the label, taking into account the nature of the drug repackaged, any packaging and expiration dating information in the manufacturer's product labeling, the characteristics of the containers, and the storage conditions to which the article may be subjected. Repackaged dosage forms must bear on their labels expiration dates as determined from information in the product labeling (see *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling*). Each single-unit or unit-dose container bears a separate label, unless the device holding the unit-dose form does not allow for the removal or separation of the intact single-unit or unit-dose container therefrom.

Storage

Store the repackaged article in a humidity-controlled environment and at the temperature specified in the individual monograph or in the product labeling. For further directions, see *Packaging and Storage Requirements* ⟨659⟩.

A refrigerator or freezer shall not be considered to be a humidity-controlled environment. Drugs that are to be stored at a cold temperature in a refrigerator or freezer must be protected during storage in the refrigerator or freezer. An outer container may be necessary for such protection; it is recommended that the drug monograph be referenced for storage.

Reprocessing

Reprocessing of repackaged unit-dose containers (i.e., removing a dosage unit from one unit-dose container and placing it in another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided that the original expiration date is maintained.

CUSTOMIZED PATIENT MEDICATION PACKAGES

In lieu of dispensing two or more prescribed drug products in separate containers, a pharmacist may, with the consent of the patient, the patient's caregiver, or a prescriber, provide a customized patient medication package (patient med pak).[3]

A patient med pak, i.e., a package prepared by a pharmacist for a specific patient, comprises a series of containers and contains two or more prescribed solid oral dosage forms. The patient med pak is so designed, or each container is so labeled, as to indicate the day and time, or period of time, that the container contents are to be taken.

It is the responsibility of the dispenser to instruct the patient or caregiver on the use of the patient med pak.

Label

The patient med pak shall bear a label stating the following:

1. The name of the patient;
2. A serial number for the patient med pak itself and a separate identifying serial number for each of the prescription orders for each of the drug products contained therein;
3. The name, strength, physical description or identification, and total quantity of each drug product contained therein;
4. The directions for use and cautionary statements, if any, contained in the prescription order for each drug product therein;
5. Any storage instructions or cautionary statements required by the official compendia;
6. The name of the prescriber of each drug product;
7. The date of preparation of the patient med pak and the beyond-use date or period of time assigned to the patient med pak (such beyond-use date or period of time shall be not longer than the shortest recommended beyond-use date for any dosage form included therein or not longer than 60 days from the date of preparation of the patient med pak, and shall not exceed the shortest expiration date on the original manufacturer's bulk containers for the dosage forms included therein); alternatively, the package label shall state the date of the prescription(s) or the date of preparation of the patient med pak, provided the package is accompanied by a record indicating the start date and the beyond-use date;
8. The name, address, and telephone number of the dispenser (and the dispenser's registration number where necessary); and
9. Any other information, statements, or warnings required for any of the drug products contained therein.

If the patient med pak allows for the removal or separation of the intact containers therefrom, each individual container shall bear a label identifying each of the drug products contained therein.

[3] It should be noticed that for patient med paks there is no special exemption from the requirements of the Poison Prevention Packaging Act. Thus, the patient med pak, if it does not meet child-resistant standards, shall be placed in an outer package that does comply, or the necessary consent of the purchaser or physician to dispense in a container not intended to be child-resistant shall be obtained.

Labeling

The patient med pak shall be accompanied by a patient package insert, in the event that any medication therein is required to be dispensed with such insert as accompanying labeling. Alternatively, such required information may be incorporated into a single, overall educational insert provided by the pharmacist for the total patient med pak.

Packaging

In the absence of more stringent packaging requirements for any of the drug products contained therein, each container of the patient med pak shall comply with the moisture permeation requirements for a Class B single-unit or unit-dose container (see *Containers—Performance Testing* ⟨671⟩). Each container shall be either nonreclosable or so designed as to show evidence of having been opened.

Guidelines

It is the responsibility of the dispenser, when preparing a patient med pak, to take into account any applicable compendial requirements or guidelines and the physical and chemical compatibility of the dosage forms placed within each container, as well as any therapeutic incompatibilities that may attend the simultaneous administration of the medications. In this regard, pharmacists are encouraged to report to USP headquarters any observed or reported incompatibilities. Once a medication has been placed in a patient med pak with another solid dosage form, it may not be returned to stock, redistributed, or resold if unused.

Recordkeeping

In addition to any individual prescription filing requirements, a record of each patient med pak shall be made and filed. Each record shall contain, as a minimum:

1. The name and address of the patient;
2. The serial number of the prescription order for each drug product contained therein;
3. The name of the manufacturer or labeler and lot number for each drug product contained therein;
4. Information identifying or describing the design, characteristics, or specifications of the patient med pak sufficient to allow subsequent preparation of an identical patient med pak for the patient;
5. The date of preparation of the patient med pak and the beyond-use date that was assigned;
6. Any special labeling instructions; and
7. The name or initials of the pharmacist who prepared the patient med pak.

GLOSSARY

Dispenser: A dispenser is a licensed or registered practitioner who is legally responsible for providing the patient with a preparation that is in compliance with prescription or a medication order and contains a specific patient label. In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. Dispensers are governed by the board of pharmacy of the individual state.

Package: The term "package" is synonymous with the term "container". See *Packaging and Storage Requirements* ⟨659⟩.

Pharmacy: A pharmacy is an establishment that is legally responsible for providing the patient with a drug preparation with a specific patient label, in compliance with a prescription or a medication order. The terms "dispenser" and "pharmacy" are used interchangeably.

Repackaging: Repackaging is the act of removing a preparation from its original primary container and placing it into another primary container, usually of smaller size.

Repackager: A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice regulations in 21 CFR 210 and 211.

⟨1151⟩ PHARMACEUTICAL DOSAGE FORMS

GENERAL CONSIDERATIONS

This chapter provides general descriptions of and definitions for drug products, or dosage forms, commonly used to administer the drug substance (active pharmaceutical ingredient, API). It discusses general principles involved in the manufacture or compounding of these dosage forms. A glossary is provided as a nomenclature resource.

A dosage form is a combination of drug substance(s) and/or excipient(s) to facilitate dosing, administration, and delivery of the medicine to the patient. The design, materials, manufacturing, and testing of all dosage forms target drug product quality.[1] A testing protocol must consider not only the physical, chemical, and biological properties of the dosage form as appropriate, but also the administration route and desired dosing regimen. The interrelationships of dosage forms and routes of administration have been summarized in the compendial taxonomy for pharmaceutical dosage forms (see *Figure 1*).[2] The organization of this general information chapter is mainly focused on the physical attributes of each particular dosage form (*Tier Two*), generally without specific reference to the route of administration. Those dosage form terms with asterisk notation (*) are not preferred and should not be used for new drug product titles. Information specific to the route of administration is given when needed.

TIER ONE
ROUTE OF ADMINISTRATION

INJECTION/IMPLANTATION GASTRO-INTESTINAL TOPICAL/DERMAL MUCOSAL INHALATION

TIER TWO
DOSAGE FORM

AEROSOLS CAPSULES CREAMS EMULSIONS FILMS FOAMS GASES GELS
GRANULES GUMS IMPLANTS INJECTIONS INSERTS IRRIGATIONS LIQUIDS LOTIONS
LOZENGES OINTMENTS PASTES PELLETS PILLS PLASTERS* POWDERS
SOAPS/SHAMPOOS SOLUTIONS SPRAYS STRIPS SUPPOSITORIES
SUSPENSIONS SYSTEMS TABLETS TAPES*

TIER THREE
RELEASE PATTERN

IMMEDIATE EXTENDED DELAYED

Figure 1. Compendial taxonomy for pharmaceutical dosage forms.

Tests to ensure compliance with *USP* standards for dosage form performance fall into one of the following areas.

Dose Uniformity

(see also *Uniformity of Dosage Units* ⟨905⟩): Consistency in dosing for a patient or consumer requires that the variation in the drug substance content of each dosage unit be accurately controlled throughout the manufactured batch or compounded lot of drug product. Uniformity of dosage units typically is demonstrated by one of two procedures: content uniformity or weight variation. The procedure for content uniformity requires the appropriate assay of the drug substance content of individual units. The procedure for weight variation uses the weight of the individual units to estimate their content. Weight variation may be used where the underlying distribution of the drug substance in the blend is presumed to be uniform and well-controlled, as in solutions. In such cases, the content of the drug substance may be adequately estimated by the net weight. Content uniformity does not rely on the assumption of blend uniformity and can be applied in all cases. Successful development and manufacture of dosage forms requires careful evaluation of the drug substance particle or droplet size, incorporation techniques, and excipient properties.

Stability

Drug product stability involves the evaluation of chemical stability, physical stability, and performance over time. The chemical stability of the drug substance in the dosage form matrix must support the expiration dating for the commercially prepared dosage forms and a beyond-use date for a compounded dosage form. Test procedures for potency must be stability indicating (see *Validation of Compendial Procedures* ⟨1225⟩). Degradation products should be quantified. In the case of dispersed or emul-

[1] In the United States, a drug with a name recognized in *USP–NF* must comply with compendial identity standards or be deemed adulterated, misbranded, or both. To avoid being deemed adulterated, such drugs must also comply with compendial standards for strength, quality, and purity, unless labeled to show all respects in which the drug differs. See the Federal Food, Drug, and Cosmetic Act (FDCA), Sections 501(b) and 502(e)(3)(b), and Food and Drug Administration (FDA) regulations at 21 CFR 299.5. In addition, to avoid being deemed misbranded, drugs recognized in *USP–NF* must also comply with compendial standards for packaging and labeling, FDCA Section 502(g). "Quality" is used herein as suitable shorthand for all such compendial requirements. This approach is also consistent with U.S. and FDA participation in the International Conference on Harmonization (ICH). The ICH guideline on specifications, Q6A, notes that "specifications are chosen to confirm the quality of the drug substance and drug product…" and defines "quality" as "The suitability of either a drug substance or drug product for its intended use. This term includes such attributes as identity, strength, and purity."

[2] Marshall K, Foster TS, Carlin HS, & Williams RL. Development of a compendial taxonomy and glossary for pharmaceutical dosage forms. *Pharm Forum.* 2003;29(5):1742–1752.

sified systems, consideration must be given to the potential for settling or separation of the formulation components. Any physical changes to the dosage form must be easily reversed (e.g., by shaking) prior to dosing or administration. For tablets, capsules, oral suspensions, and implants, in vitro release test procedures such as dissolution and disintegration provide a measure of continuing consistency in performance over time (see *Dissolution* 〈711〉, *Disintegration* 〈701〉, and *Drug Release* 〈724〉).

Bioavailability

(see also *In Vitro and In Vivo Evaluation of Dosage Forms* 〈1088〉 and *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* 〈1090〉): Bioavailability is influenced by factors such as the method of manufacture or compounding, particle size, crystal form (polymorph) of the drug substance, the properties of the excipients used to formulate the dosage form, and physical changes as the drug product ages. Assurance of consistency in bioavailability over time (bioequivalence) requires close attention to all aspects of the production (or compounding) and testing of the dosage form. With proper justification, in vitro release testing (e.g., disintegration and dissolution) may be used as a surrogate to demonstrate consistent availability of the drug substance from the formulated dosage.

Manufacture

Although detailed instructions about the manufacture of any of these dosage forms are beyond the scope of this general information chapter, general manufacturing principles have been included.[3] Information relative to extemporaneous compounding of dosage forms can be found in *Pharmaceutical Compounding—Nonsterile Preparations* 〈795〉 and *Pharmaceutical Compounding—Sterile Preparations* 〈797〉.

Route of Administration

The primary routes of administration for pharmaceutical dosage forms can be defined as parenteral (see *Injections and Implanted Drug Products* 〈1〉), gastrointestinal (see *Oral Drug Products—Product Quality Tests* 〈2〉), topical/dermal (see *Topical and Transdermal Drug Products—Product Quality Tests* 〈3〉), mucosal, and inhalation (see *Inhalation and Nasal Drug Products—General Information and Product Quality Tests* 〈5〉), and each has subcategories as needed. Many tests used to ensure quality generally are applied across all of the administration routes, but some tests are specific for individual routes. For example, products intended for injection must be evaluated using *Sterility Tests* 〈71〉, *Bacterial Endotoxins Test* 〈85〉, or *Pyrogen Test* 〈151〉, and the manufacturing process (and sterilization technique) employed for parenterals (by injection) should ensure compliance with these tests. Tests for particulate matter may be required for certain dosage forms depending on the route of administration (e.g., by injection—*Particulate Matter in Injections* 〈788〉, or mucosal—*Particulate Matter in Ophthalmic Solutions* 〈789〉). Additionally, dosage forms intended for the inhalation route of administration must be monitored for particle size and spray pattern (for a metered-dose inhaler or dry powder inhaler) and droplet size (for nasal sprays). Further information regarding administration routes and suggested testing can be found in the *Guide to General Chapters, Charts 4–8, 10,* and *13*.

An appropriate manufacturing process and testing regimen help ensure that a dosage form can meet the appropriate quality attributes for the intended route of administration.

Packaging and Storage

Suitable packaging is determined for each product. For additional information about meeting packaging requirements listed in the individual labeling, refer to *Packaging and Storage Requirements* 〈659〉, *Containers—Performance Testing* 〈671〉, *Good Packaging Practices* 〈1177〉, and *Good Repackaging Practices* 〈1178〉. Product labeling must specify storage requirements that describe environmental conditions, limitations, and restrictions. For instance, exposure to excessive temperature, humidity, and light can influence the ability of the packaging to protect the product.

Labeling Statements

Some dosage forms or articles have mandatory labeling statements that are given in the Code of Federal Regulations (e.g., 21 CFR 201.320 and 21 CFR 369.21). The text of 21 CFR should be consulted to determine the current recommendations.

Change to read:

PRODUCT QUALITY TESTS, GENERAL

ICH Guidance Q6A (available at www.ich.org) recommends specifications (list of tests, references to analytical procedures, and acceptance criteria) to ensure that drug products are safe and effective at the time of release and over their shelf life. Tests

[3] The terms "manufacture" and "preparation" are used interchangeably in this general chapter.

that are universally applied to ensure safety, efficacy, strength, quality, and purity include description, identification, assay, and impurities.

Description

The *Definition* section (see *General Notices and Requirements* 4.10) in a *USP* monograph describes the drug product and specifies the range of acceptable assayed content of the drug substance(s) present in the dosage form. For certain products, the *Definition* includes any relevant additional information, such as the presence or absence of other components, excipients, or adjuvants, cautionary statements on toxicity and stability, etc. While appearance information to aid in identification is used in a regulatory submission (e.g., a qualitative description of size, shape, color, etc.) it is typically not required as part of a *USP* monograph. This information is drug product specific.

Identification

Identification tests are discussed in the *General Notices and Requirements* 5.40. Identification tests should establish the identity of the drug substance(s) present in the drug product and should discriminate between compounds of closely related structure that are likely to be present. Identification tests should be specific for the drug substance(s). For example, the infrared absorption spectrum is often used (see *Mid-Infrared Spectroscopy* ⟨854⟩ and *Spectrophotometric Identification Tests* ⟨197⟩). If no suitable infrared spectrum can be obtained, other analytical methods can be used. Near-infrared (NIR) or Raman spectrophotometric methods could also be acceptable as the sole identification method of the drug product formulation (see *Near-Infrared Spectroscopy* ⟨1119⟩ and *Raman Spectroscopy* ⟨1120⟩). Identification by a chromatographic retention time from a single procedure is not regarded as specific. The use of retention times from two chromatographic procedures for which the separation is based on different principles or a combination of tests in a single procedure can be acceptable (see *Chromatography* ⟨621⟩ and *Thin-Layer Chromatographic Identification Test* ⟨201⟩).

Assay

A specific and stability-indicating test should be used to determine the strength (drug substance content) of the drug product. Some examples of these procedures are *Antibiotics—Microbial Assays* ⟨81⟩, ⟨621⟩, or *Assay for Steroids* ⟨351⟩. In cases when the use of a nonspecific assay is justified (e.g., *Titrimetry* ⟨541⟩), other supporting analytical procedures should be used to achieve specificity. When evidence of excipient interference with a nonspecific assay exists, a procedure with demonstrated specificity should be used.

Impurities

Process impurities, synthetic by-products, and other inorganic and organic impurities may be present in the drug substance and excipients used in the manufacture of the drug product. These impurities are evaluated by tests in the drug substance and excipients monographs. Impurities arising from degradation of the drug substance or from the drug-product manufacturing process should be monitored. *Residual Solvents* ⟨467⟩ is applied to all products where relevant.

In some cases, testing for heavy metal impurities is appropriate. ●● (Official 1-Jan-2018)

In addition to the universal tests listed, the following tests may be considered on a case-by-case basis.

Physicochemical Properties

Examples include *pH* ⟨791⟩, *Viscosity—Capillary Methods* ⟨911⟩ or *Viscosity—Rotational Methods* ⟨912⟩, and *Specific Gravity* ⟨841⟩.

Particle Size

For some dosage forms, particle size can have a significant effect on dissolution rates, bioavailability, therapeutic outcome, and stability. Procedures such as those found in ●*Inhalation and Nasal Drug Products: Aerosols, Sprays, and Powders—Performance Quality Tests* ⟨601⟩● (CN 1-May-2017) and *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩ could be used.

Uniformity of Dosage Units

See the discussion of *Dose uniformity* in the *General Considerations* section.

Water Content

A test for water content is included when appropriate (see *Water Determination* ⟨921⟩).

Microbial Limits

The type of microbial test(s) and acceptance criteria are based on the nature of the nonsterile drug product, method of manufacture, and the route of administration (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* ⟨61⟩, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* ⟨62⟩, and *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩).

Antimicrobial Preservative Content

Acceptance criteria for preservative content in multidose products should be established. They are based on the levels of antimicrobial preservative necessary to maintain the product's microbiological quality at all stages throughout its proposed usage and shelf life (see *Antimicrobial Effectiveness Testing* ⟨51⟩).

Antioxidant Content

If antioxidants are present in the drug product, tests of their content should be performed to maintain the product's quality at all stages throughout its proposed usage and shelf life.

Sterility

Depending on the route of administration (e.g., ophthalmic preparations, implants, aqueous-based preparations for oral inhalation, and injections) sterility of the product is demonstrated as appropriate (see ⟨71⟩).

Dissolution

A test to measure the release of the drug substance(s) from the drug product normally is included for dosage forms such as tablets, capsules, suspensions, granules for suspensions, implants, transdermal delivery systems, and medicated chewing gums. Single-point measurements typically are used for immediate-release dosage forms. For modified-release dosage forms, appropriate test conditions and sampling procedures are established as needed (see ⟨711⟩ and ⟨724⟩). In some cases, dissolution testing may be replaced by disintegration testing (see ⟨701⟩).

Breaking Force and Friability

These parameters are evaluated as in-process controls. Acceptance criteria depend on packaging, supply chain, and intended use (see *Tablet Friability* ⟨1216⟩ and *Tablet Breaking Force* ⟨1217⟩).

Leachables

When evidence exists that leachables from the container–closure systems (e.g., rubber stopper, cap liner, or plastic bottle) have an impact on the safety or efficacy of the drug product, a test is included to evaluate the presence of leachables.

Other Tests

Depending on the type and composition of the dosage form, other tests such as alcohol content, redispersibility, particle size distribution, rheological properties, reconstitution time, endotoxins/pyrogens, particulate matter, functionality testing of delivery systems, delivered dose uniformity, viscosity, and osmolarity may be necessary.

DOSAGE FORMS

Aerosols

Aerosols are dosage forms packaged under pressure and contain therapeutic agent(s) and a propellant that are released upon actuation of an appropriate valve system. Upon actuation of the valve system, the drug substance is released as a plume of fine particles or droplets. Only one dose is released from the preparation upon actuation of a metered valve. In the case of topical products and depending on the nature of the drug substance and the conditions being treated, actuation of the valve may result in a metered release of a controlled amount of the formulation or the continuous release of the formulation as long as the valve is depressed.

The aerosol dosage form refers only to those products packaged under pressure that release a fine mist of particles or droplets when actuated (see *Glossary*). Other products that produce dispersions of fine droplets or particles will be covered in subsequent sections (e.g., *Powders* and *Sprays*).

TYPICAL COMPONENTS

Typical components of aerosols are the formulation containing one or more drug substance(s) and propellant, the container, the valve, and the actuator. Each component plays a role in determining various characteristics of the emitted plume, such as droplet or particle size distribution, uniformity of delivery of the therapeutic agent, delivery rate, and plume velocity and geometry. The metering valve and actuator act in tandem to generate the plume of droplets or particles. The metering valve delivers an accurate volume of the pressurized liquid formulation from the container. The actuator directs the metered volume to a small orifice that is open to the atmosphere. Upon actuation, the formulation is forced through the opening, forming the fine mist of particles that are directed to the site of administration.

Aerosol preparations may consist of either a two-phase (gas and liquid) or a three-phase (gas, liquid, and solid or liquid) formulation. The two-phase formulation consists of drug substance(s) dissolved in liquefied propellant. Co-solvents such as alcohol may be added to enhance the solubility of the drug substance(s). Three-phase inhalation and nasal aerosol systems consist of suspended drug substance(s) in propellant(s), co-solvents, and potentially other suitable excipients. The suspension or emulsion of the finely divided drug substance is typically dispersed in the liquid propellant with the aid of suitable biocompatible surfactants or other excipients.

Propellants for aerosol formulations are typically low molecular weight hydrofluorocarbons or hydrocarbons that are liquid when constrained in the container, exhibit a suitable vapor pressure at room temperature, and are biocompatible and nonirritating. Compressed gases do not supply a constant pressure over use and typically are not used as propellants.

Metal containers can withstand the vapor pressure produced by the propellant. Excess formulation may be added to the container to ensure that the full number of labeled doses can be accurately administered. The container and closure must be able to withstand the pressures anticipated under normal use conditions as well as when the system is exposed to elevated temperatures.

TYPES OF AEROSOL DOSAGE FORMS

Aerosol dosage forms can be delivered via various routes. The container, actuator, and metering valve, as well as the formulation, are designed to target the site of administration.

Inhalation aerosols, commonly known as metered-dose inhalers (MDIs), are intended to produce fine particles or droplets for inhalation through the mouth and deposition in the pulmonary tree. The design of the delivery system is intended to release measured mass and appropriate quality of the active substance with each actuation.

Nasal aerosols, commonly known as nasal MDIs, produce fine particles or droplets for delivery through the nasal vestibule and deposition in the nasal cavity. Each actuation of the valve releases a measured mass of the drug substance with appropriate quality characteristics.

Lingual aerosols are intended to produce fine particles or droplets for deposition on the surface of the tongue. The design of the delivery system releases one dose with each actuation.

Topical aerosols produce fine particles or droplets for application to the skin.

LABELING FOR PROPER USE

Refer to 21 CFR 201.320 and 21 CFR 369.21.

Capsules

Capsules are solid dosage forms in which the drug substance and/or excipients are enclosed within a soluble container or shell or coated on the capsule shell. The shells may be composed of two pieces (a body and a cap), or they may be composed of a single piece. Two-piece capsules are commonly referred to as hard-shell capsules, and one-piece capsules are often referred to as soft-shell capsules. This two-piece and one-piece capsule distinction, although imprecise, reflects differing levels of plasticizers in the two compositions and the fact that one-piece capsules typically are more pliable than two-piece capsules.

The shells of capsules are usually made from gelatin. However, they may also be made from cellulose polymers or other suitable material. Most capsules are designed for oral administration. When no deliberate effort has been made to modify the drug substance release rate, capsules are referred to as immediate-release.

TWO-PIECE OR HARD-SHELL CAPSULES

Two-piece capsules consist of two telescoping cap and body pieces in a range of standard sizes.

ONE-PIECE OR SOFT-SHELL CAPSULES

One-piece capsules typically are used to deliver a drug substance as a solution or suspension. Liquid formulations placed into one-piece capsules may offer advantages by comparison with dry-filled capsules and tablets in achieving content uniformity of potent drug substance(s) or acceptable dissolution of drug substance(s) with poor aqueous solubility. Because the contact between the shell wall and its liquid contents is more intimate than in dry-filled capsules, undesired interactions may be more likely to occur (including gelatin crosslinking and pellicle formation).

MODIFIED-RELEASE CAPSULES

The release of drug substance(s) from capsules can be modified in several ways. There are two categories of modified-release capsule formulations recognized by USP.

Delayed-release capsules: Capsules are sometimes formulated to include enteric-coated granules to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation. Enteric-coated multiparticulate capsule dosage forms may reduce variability in bioavailability associated with gastric emptying times for larger particles (i.e., tablets) and to minimize the likelihood of a therapeutic failure when coating defects occur during manufacturing. Alternatively, a coating may be applied to the capsule shell to achieve delayed release of the contents.

Extended-release capsules: Extended-release capsules are formulated in such a manner as to make the contained drug substance available over an extended period of time following ingestion. Expressions such as "prolonged-action", "repeat-action", "controlled-release", and "sustained-release" have also been used to describe such dosage forms. However, the term "extended-release" is used for pharmacopeial purposes. Requirements for dissolution (see 〈711〉) are typically specified in the individual monograph.

Methods for modifying drug substance release from capsules include coating the filled capsule shells or the contents, in the case of dry-filled capsules.

PREPARATION

Two-piece capsules: Two-piece gelatin capsules are usually formed from blends of gelatins that have relatively high gel strength in order to optimize shell clarity and toughness or from hypromellose. They may also contain colorants such as D&C and FD&C dyes[4] or various pigments, opaquing agents such as titanium dioxide, dispersing agents, plasticizers, and preservatives. Gelatin capsule shells normally contain between 12% and 16% water.

The shells are manufactured in one set of operations and later filled in a separate manufacturing process. Two-piece shell capsules are made by a process that involves dipping shaped pins into gelatin or hypromellose solutions, followed by drying, cutting, and joining steps.

Powder formulations for two-piece gelatin capsules generally consist of the drug substance and at least one excipient. Both the formulation and the method of filling can affect release of the drug substance. In the filling operation, the body and cap of the shell are separated before filling. Following the filling operation, the machinery rejoins the body and cap and ensures satisfactory closure of the capsule by exerting appropriate force on the two pieces. The joined capsules can be sealed after filling by a band at the joint of the body and cap or by a designed locking joint between the cap and body. In compounding prescription practice, two-piece capsules may be hand-filled. This permits the prescriber the choice of selecting either a single drug substance or a combination of drug substances at the exact dose level considered best for an individual patient.

One-piece capsules: One-piece capsules are formed, filled, and sealed in a single process on the same machine and are available in a wide variety of sizes, shapes, and colors. The most common type of one-piece capsule is that produced by a rotary die process that results in a capsule with a seam. The soft gelatin shell is somewhat thicker than that of two-piece capsules and is plasticized by the addition of polyols such as glycerin, sorbitol, or other suitable materials. The ratio of the plasticizer to the gelatin can be varied to change the flexibility of the shell depending on the nature of the fill material, its intended usage, or environmental conditions.

In most cases, one-piece capsules are filled with liquids. Typically, drug substances are dissolved or suspended in a liquid vehicle. Classically, an oleaginous vehicle such as a vegetable oil was used. However, nonaqueous, water-miscible liquid vehicles such as the lower molecular weight polyethylene glycols are now more common. The physicochemical properties of the vehicle can be chosen to ensure stability of the drug substance as well as to influence the release profile from the capsule shell.

[4] In 1960 Congress enacted the Color Additive Amendments, requiring the FDA to regulate dyes, pigments, or other coloring agents in foods, drugs, and cosmetics separately from food additives. Under the law, color additives are deemed unsafe unless they are used in compliance with FDA regulations. The law provides a framework for the listing and certification of color additives. See FDCA section 721; see FDA regulations at 21 CFR Part 70. Colors must also be listed in pertinent FDA regulations for specific uses; the list of color additives for drugs that are exempt from certification is published at 21 CFR Part 73, Subpart B. FDA also conducts a certification program for batches of color additives that are required to be certified before sale; see 21 CFR Part 74 (Subpart B re: drugs). Regulations regarding certification procedures, general specifications, and the listing of certified provisionally listed colors are at 21 CFR Part 80. FDA maintains a color additives website with links to various legal and regulatory resources at:http://www.fda.gov; search by document title.

Creams

(See *Emulsions.*)

Emulsions

An emulsion is a dispersed colloidal system consisting of two immiscible liquid phases generally stabilized with one or more suitable agents.

Typical pharmaceutical emulsions are prepared from immiscible aqueous and organic (oil) liquids. Complex multiple-phase systems may exist in an emulsion. Whether the organic or the aqueous phase is the dispersed phase depends on the volumes of the two phases, the emulsifier chosen, and the method of preparation. When an oil phase is dispersed in an aqueous phase, the emulsion is termed an oil in water (O/W) emulsion and water is referred to as the continuous phase. When water is dispersed in oil, the emulsion is referred to as a water in oil (W/O) emulsion. Emulsions have dispersed phases typically ranging from 0.1 to 100 µm. Emulsions are opaque while microemulsions are usually transparent or translucent. Microemulsions have dispersed phases less than 0.1 µm.

Emulsions may exhibit three types of instability: flocculation, creaming, and coalescence. Flocculation describes the process by which the dispersed phase comes out of suspension in the form of flakes. Coalescence is another form of instability—small droplets within the media continuously combine to form progressively larger droplets. Emulsions can also undergo creaming, where one of the phases migrates to the top (or the bottom, depending on the relative densities of the two phases) of the emulsion. To prevent flocculation, creaming, and coalescence of the emulsions, manufacturers commonly add surfactants, pH-modifying agents, or emulsifying agents to increase the stability of emulsions so that the emulsion does not change significantly with time.

Emulsions are widely used as pharmaceutical dosage forms. Oral emulsions have been prepared to improve taste, solubility, stability, or bioavailability. Emulsions for topical administration are referred to as creams, lotions, and sometimes ointments. Parenteral emulsions have been used for anaesthetics, parenteral nutrition, and to deliver poorly water-soluble drugs.

CREAMS

Creams are semisolid emulsion dosage forms. They often contain more than 20% water and volatiles, and/or typically contain less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or to the mucous membranes. Creams have a relatively soft, spreadable consistency and can be formulated as either a water-in-oil emulsion (e.g., *Cold Cream* or *Fatty Cream* as in the *European Pharmacopoeia*) or as an oil-in-water emulsion (e.g., *Betamethasone Valerate Cream*). Creams are generally described as either nonwashable or washable, reflecting the fact that an emulsion with an aqueous external continuous phase is more easily removed than one with a nonaqueous external phase (water-in-oil emulsion).

LOTIONS

Lotions are an emulsified liquid dosage form intended for external application to the skin. Historically, some topical suspensions such as calamine lotion have been called lotions but that nomenclature is not currently preferred. Lotions share many characteristics with creams. The distinguishing factor is that they are more fluid than semisolid and thus pourable. Due to their fluid character, lotions are more easily applied to large skin surfaces than semisolid preparations. Lotions may contain antimicrobial agents as preservatives.

INJECTABLE EMULSIONS

Injectable emulsions are sterile liquid dosage forms of drug substances dissolved or dispersed in a suitable emulsion medium. Injectable emulsions are for parenteral administration of poorly water-soluble drugs.

OINTMENTS

Ointments are sometimes semisolid emulsion dosage forms (see *Ointments*).

PREPARATION

Chapter ⟨795⟩ provides general information regarding the preparation of emulsions.

Creams: Creams may be formulated from a variety of oils, both mineral and vegetable, and from fatty alcohols, fatty acids, and fatty esters. Emulsifying agents include nonionic surfactants, detergents, and soaps. Soaps are usually formed in situ during the preparation of creams from a fatty acid in the oil phase hydrolyzed by a base dissolved in the aqueous phase.

General Chapters

Preparation usually involves separating the formula components into two portions: lipid and aqueous. The lipid portion contains all water-insoluble components and the aqueous portion contains the water-soluble components. Both phases are heated to a temperature above the melting point of the highest melting component. The phases are then mixed and the mixture is stirred until reaching ambient temperature or until the mixture has congealed. Mixing is generally continued during the cooling process to promote uniformity. Traditionally, the aqueous phase is added to the lipid phase, but comparable results have been obtained with the reverse procedure. High-shear homogenization may be employed to reduce particle or droplet size and to improve the physical stability of the resultant dosage form.

The drug substance(s) can be added to the phase in which it is soluble at the beginning of the manufacturing process, or it can be added after the cream is prepared by a suitable dispersion process such as levigation or milling with a roller mill. Creams usually require the addition of a preservative(s) unless they are compounded immediately prior to use and intended to be consumed in a relatively short period of time.

Lotions: Lotions are usually prepared by dissolving or dispersing the drug substance into the more appropriate phase (oil or water), adding the appropriate emulsifying or suspending agents, and mixing the oil and water phases to form a uniform fluid emulsion.

Injectable emulsions: ⟨1⟩ provides guidance on sterile preparations. Emulsions intended for parenteral administration can be formulated using the same principles as creams and lotions. The formulation should be designed for ease of administration. The particle size of the dispersed phase can vary by route of administration. For example, emulsions intended for intravenous administration should comply with *Globule Size Distribution in Lipid Injectable Emulsions* ⟨729⟩. The procedure to assure sterility should be validated by media fills. Preservatives are generally not used in injectable emulsions.

Ointments: (See *Ointments*.)

Films

Films are thin sheets that are placed in the oral cavity. They contain one or more layers. A layer may or may not contain the drug substance. Typically, these thin sheets are formed by casting or extrusion which results in a dispersion of the components through the film. Films are classified by the site of application. "Oral films" can be formulated to deliver medication to the mouth such as oral hygiene products or to deliver medication to the gastrointestinal tract for absorption. "Buccal films" and "sublingual films" are formulated to facilitate absorption through the proximal mucosal membranes avoiding first pass metabolism or degradation in the gastrointestinal tract and providing a quick onset of action.

Films can be formulated with edible polymers such as pullulan or with water-soluble polymers such as modified cellulose, edible gums, and copolymers. The dissolution rate of the film is controlled to facilitate incorporation of the medication into saliva or for absorption by the proximal mucosa. These films must be substantial enough to maintain their integrity during manufacture and packaging, and permit handling by the patient. Because of the rapid dissolution, taste and mouth feel are important considerations.

Foams

Foams are preparations that comprise gas bubbles distributed in a liquid. The liquid contains the drug substance and suitable excipients. Medicated foams may be packaged in pressurized containers or in other special dispensing devices. For medicated foams dispensed from nonpressurized containers, the use of mechanical force is required to generate the foam. Foams are intended for application to the skin or mucous membranes. The medicated foam is formed at the time of application. Surfactants are used to ensure the distribution of the gas in the liquid and to stabilize the foam. Medicated foams have a semisolid consistency and can be formulated to quickly break down into liquid or to remain as foam to ensure prolonged contact.

Medicated foams intended to treat severely injured skin or open wounds must be sterile.

PREPARATION

A foam may contain one or more drug substances, surfactants, and aqueous or nonaqueous liquids, and is produced with or without the aid of propellants. When a propellant is not used, mechanical work is required to generate the foam. If the propellant is in the internal (discontinuous) phase, a stable foam is discharged. If the propellant is in the external (continuous) phase, a quick-breaking foam is discharged. Quick-breaking foams formulated with alcohol create a cooling sensation after application to the skin and may have antimicrobial properties.

Gases

Medical gases are products that are administered directly as a gas. A medical gas has a direct pharmacological action or acts as a diluent for another medical gas. Gases used as excipients for administration of aerosol products, as an adjuvant in packaging, or produced by other dosage forms, are not included in this definition.

COMPONENTS

Medical gases may be single components or defined mixtures of components. Mixtures can also be extemporaneously prepared at the point of use.

ADMINISTRATION

Medical gases may be administered to the patient using several methods: nasal cannulas, face masks, atmospheric tents, and endotracheal tubes for the pulmonary route; hyperbaric chambers for the pulmonary and dermal routes of administration; jetted tubes that are directed at dental tissue to promote drying in preparation for fillings and crowns; tubes for expanding the intestines to facilitate medical imaging during colonoscopy; tubes for expanding the pelvis via transuterine inflation in preparation for fallopian tubal ligation; and tubes for expanding angioplasty devices. The dose of medical gas is typically metered by a volume rate of flow under ambient temperature and pressure conditions. Administration of a highly compressed gas generally requires a regulator to decrease the pressure, a variable-volume flow controller, and suitable tubing to conduct the gas to the patient. For pulmonary administration, the gas flow will be directed to the nose or mouth by a suitable device or into the trachea through a mechanical ventilator. When medical gases are administered chronically, provision for humidification is common. Care should be exercised to avoid microbial contamination.

SPECIAL CONSIDERATIONS

The container and system fittings should be appropriate for the medical gas. Adaptors should not be used to connect containers to patient-use supply system piping or equipment. Large quantities of gases such as oxygen or nitrogen can be stored in the liquid state in a cryogenic container and converted into a gas, as needed, by evaporation. Additional rules concerning the construction and use of cryogenic containers are promulgated by governmental agencies (e.g., U.S. Department of Commerce).

Containers, tubing, and administration masks employed for gases containing oxygen are free of any compound that would be sensitive to oxidation or that would be irritating to the respiratory tract.

A significant fraction of the dose of a medical gas may be released into the general vicinity of the patient due to incomplete absorption. Adequate ventilation may be necessary to protect health care workers and others from exposure to the gas (e.g., nitrous oxide).

Gels

Gels are semisolids consisting either of suspensions of small inorganic particles or of organic molecules interpenetrated by a liquid. Jellies are a type of gel that typically have a higher water content. Gels can be classed either as single-phase or two-phase systems.

A two-phase gel consists of a network of small discrete particles (e.g., *Aluminum Hydroxide Gel* or *Psyllium Hemicellulose*). These gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation. They should be shaken before use to ensure homogeneity and should be so labeled.

Single-phase gels consist of organic macromolecules uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid. Single-phase gels may be made from natural or synthetic macromolecules (e.g., *Carbomer, Hypromellose,* or *Starch*) or natural gums (e.g., *Tragacanth*). The latter preparations are also called mucilages. Although these gels are commonly aqueous, alcohols and oils may be used as the continuous phase.

Gels can be administered by the topical or mucosal routes. In veterinary medicine, gels can be administered via mammary infusion.

PREPARATION

See ⟨795⟩ for general procedures. Also see the information contained under *Suspensions* for the formulation and manufacture of gels containing inorganic components or drug substances in the solid phase. See ⟨797⟩ for general procedures for the preparation of sterile gels such as *Lidocaine Hydrochloride Jelly.*

Gels formed with large organic molecules may be formed by dispersing the molecule in the continuous phase (e.g., by heating starch), by cross-linking the dispersed molecules by changing the pH (as for *Carbomer Copolymer*), or by reducing the continuous phase (as for jellies formed with sucrose).

Care should be taken to ensure uniformity of the drug substances by dispersing them by vigorous mixing or milling, or by shaking if the preparation is less viscous.

Granules

Granules are solid dosage forms that are composed of agglomerations of smaller particles. These multicomponent compositions are prepared for oral administration and are used to facilitate flexible dosing regimens as granules or as suspensions, address stability challenges, allow taste masking, or facilitate flexibility in administration (for instance, to pediatric patients, geriatric patients, or animals). Granular dosage forms may be formulated for direct oral administration and may facilitate compounding of multiple drug substances by allowing compounding pharmacists to blend various granular compositions in the retail or hospital pharmacy. More commonly, granules are reconstituted to a suspension by the addition of water or a supplied liquid diluent immediately prior to delivery to the patient. Effervescent granules are formulated to liberate gas (carbon dioxide) upon addition of water. Common examples of effervescent granules include antacid and potassium supplementation preparations. Common therapeutic classes formulated as granule dosage forms include antibiotics, certain laxatives (such as senna extract products), electrolytes, and various cough and cold remedies that contain multiple drug substances.

PREPARATION

Granules are often the precursors used in tablet compression or capsule filling. Although this application represents a pharmaceutical intermediate and not a final dosage form, numerous commercial products are based on granules. In the typical manufacture of granules, the drug substance(s) is blended with excipients (processing aids) and wetted with an appropriate pharmaceutical binding solution, solvent, or blend of solvents to promote agglomeration. This composition is dried and sized to yield the desired material properties.

Frequently, granules are used because the drug substance is unstable in aqueous environments and cannot be exposed to water for periods sufficient to accommodate manufacture, storage, and distribution in a suspension. Preparation of the liquid dosage form from the granules immediately prior to dispensing allows acceptable stability for the duration of use. Granules manufactured for this purpose are packaged in quantities sufficient for a limited time period—usually one course of therapy that typically does not exceed 2 weeks. In addition to the drug substances, other ingredients may be added to ensure acceptable stability (e.g., buffers, antioxidants, or chelating agents) or to provide color, sweetness, and flavor; and for suspensions, to provide acceptable viscosity to ensure adequate suspension of the particulate to enable uniform dosing.

Effervescent granules are typically formulated from sodium or potassium bicarbonate and an acid such as citric or tartaric acid. To prevent untimely generation of carbon dioxide, manufacturers should take special precautions to limit residual water in the product due to manufacture and to select packaging that protects the product from moisture. The manufacture of effervescent granules can require specialized facilities designed to maintain very low humidity (approximately 10% relative humidity). Effervescent powder mixtures are purposely formed into relatively course granules to reduce the rate of dissolution and provide a more controlled effervescence.

Reconstitution of granules must ensure complete wetting of all ingredients and sufficient time and agitation to allow the soluble components to dissolve. Specific instructions for reconstitution provided by the manufacturer should be carefully followed.

Reconstituted suspensions should be thoroughly mixed or shaken before use to resuspend the dispersed particulates. This is especially true of suspension preparations dosed from multiple-dose containers. For particularly viscous suspensions prone to air entrapment, instructions may advise the user how to shake the preparation to resuspend settled particulates while minimizing air entrapment.

For granules reconstituted to form suspensions for oral administration, acceptable suspension of the particulate phase depends on the particle size of the dispersed phase as well as the viscosity of the vehicle. Temperature can influence the viscosity, which influences suspension properties and the ease of removal of the dose from the bottles. In addition, temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. Thus, clear instructions should be provided regarding the appropriate storage temperature for the product.

Gums

Medicated gum is a pliable dosage form that is designed to be chewed rather than swallowed. Medicated gums release the drug substance(s) into the saliva. Medicated gums can deliver therapeutic agents for local action in the mouth or for systemic absorption via the buccal or gastrointestinal routes (e.g., nicotine or aspirin). Most gums are manufactured using the conventional melting process derived from the confectionary industry or alternatively may be directly compressed from gum powder. Medicated gums are formulated from insoluble synthetic gum bases such as polyisoprene, polyisobutylene, isobutyleneisoprene copolymer, styrene butadiene rubber, polyvinyl acetate, polyethylene, ester gums, or polyterpenes. Plasticizers and softeners such as propylene glycol, glycerin, oleic acid, or processed vegetable oils are added to keep the gum base pliable and to aid in the incorporation of the drug substance(s), sweeteners, and flavoring agents. Sugars as well as artificial sweeteners and flavorings are incorporated to improve taste, and dyes may be used to enhance appearance. Some medicated gums are coated with magnesium stearate to reduce tackiness and improve handling during packaging. A preservative may be added.

PREPARATION

Melted gum: The gum base is melted at a temperature of about 115° until it has the viscosity of thick syrup and, at that point, is filtered through a fine-mesh screen. This molten gum base is transferred to mixing tanks where the sweeteners, plasticizers, and typically the drug substance are added and mixed. Colorings, flavorings, and preservatives are added and mixed while the melted gum is cooling. The cooled mixture is shaped by extrusion or rolling and cutting. Dosage units of the desired shape and potency are packaged individually. Additional coatings such as powder coatings to reduce tackiness or film or sugar coatings may be added to improve taste or facilitate bulk packaging.

Directly compressed gum: The gum base is supplied in a free-flowing granular powder form. The powder gum base is then dry blended with sweeteners, flavors, the drug substance, and lubricant. The blend is then processed through a conventional tablet press and tableted into desired shapes. The resulting medicated gum tablets can be further coated with sugar or sugar-free excipients. These tablets can be packaged in blisters or bottles as needed.

SPECIAL CONSIDERATIONS

Medicated gums are typically dispensed in unit-dose packaging. The patient instructions also may include a caution to avoid excessive heat.

Implants

Implants are long-acting dosage forms that provide continuous release of the drug substance often for periods of months to years. They are administered by the parenteral route. For systemic delivery they may be placed subcutaneously, or for local delivery they can be placed in a specific region in the body (e.g., in the sinus, in an artery, in the eye, in the brain, etc.).

Several types of implants are available. Pellet implants are small, sterile, solid masses composed of a drug substance with or without excipients. They are usually administered by means of a suitable special injector (e.g., trocar) or by surgical incision. Release of the drug substance from pellets is typically controlled by diffusion and dissolution kinetics. The size of the pellets and rate of erosion will influence the release rate, which typically follows first-order kinetics. Drug substance release from pellets for periods of 6 months or more is possible.

Resorbable microparticles are a type of implant that provides extended release of a drug substance over periods varying from a few weeks to months. They can be administered subcutaneously or intramuscularly for systemic delivery, or they may be deposited in a desired location in the body for site-specific delivery. Injectable resorbable microparticles (or microspheres) generally range from 20 to 100 µm in diameter. They are composed of a drug substance dispersed within a biocompatible, bioresorbable polymeric excipient (matrix). Poly(lactide-co-glycolide) polymers have been used frequently. These excipients typically resorb by hydrolysis of ester linkages. The microparticles are administered by suspension in an aqueous vehicle followed by injection with a conventional syringe and needle. Release of the drug substance from the microparticles begins after physiological fluid enters the polymer matrix, dissolving some of the drug substance that is then released by a diffusion-controlled process. Drug release also can occur as the matrix erodes.

Polymer implants can be formed as a single-shaped mass such as a cylinder. The polymer matrix must be biocompatible (*see The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩), but it can be either biodegradable or nonbiodegradable. Shaped polymer implants are administered by means of a suitable special injector. Release kinetics are typically not zero-order, but zero-order kinetics are possible. Drug substance release can be controlled by the diffusion of the drug substance from the bulk polymer matrix or by the properties of a rate-limiting polymeric membrane coating. Polymer implants are used to deliver potent small molecules like steroids (e.g., estradiol for cattle) and large molecules like peptides [e.g., luteinizing hormone-releasing hormone (LHRH)]. Example durations of drug substance release are 2 and 3 months for biodegradable implants and up to 3 years for nonbiodegradable implants. An advantage of biodegradable implants is that they do not require removal after the release of all drug substance content. Nonbiodegradable polymer implants can be removed before or after a drug substance release is complete or may be left in situ. An implant can have a tab with a hole in it to facilitate suturing it in place (e.g., for an intravitreal implant for local ocular delivery). Such implants may provide therapeutic release for periods as long as 2.5 years.

Drug substance-eluting stents combine the mechanical effect of the stent to maintain arterial patency with the prolonged pharmacologic effect of the incorporated drug substance (to reduce restenosis, inhibit clot formation, or combat infection). As an example, a metal stent can be coated with a nonbiodegradable or biodegradable polymer-containing drug substance. The resultant coating is a polymeric matrix that controls the extended release of the drug substance.

In veterinary medicine, drug substance(s) in pellets may be implanted subcutaneously in the animal's ear (cattle).

PREPARATION

Pellet implants are made by drug substance compression or molding. Cylindrical polymeric implants are typically made by melt extrusion of a blend of drug substance and polymer, resulting in a rod that is cut into shorter lengths. Polymer implants

can also be made by injection molding. Still other implants are assembled from metal tubes and injection-molded plastic components.

Sterility can be achieved by terminal sterilization or by employing aseptic manufacturing procedures.

Injections

(See *Emulsions, Powders, Solutions,* and *Suspensions.*)

Injections are not treated as a dosage form in this chapter. ⟨1⟩ provides quality and other information about injectable products. Information on specific dosage form terminology can be found in the *Glossary.* For appropriate injection nomenclature, see *Nomenclature* ⟨1121⟩.

Excess volume in injections: Each container of an injection is filled with a volume in slight excess of the labeled "size" or the volume that is to be withdrawn. The excess volumes recommended in *Table 1* are usually sufficient to permit withdrawal and administration of the labeled volumes.

Table 1

Labeled Size (mL)	Recommended Excess Volume For Mobile Liquids (mL)	For Viscous Liquids (mL)
0.5	0.10	0.12
1.0	0.10	0.15
2.0	0.15	0.25
5.0	0.30	0.50
10.0	0.50	0.70
20.0	0.60	0.90
30.0	0.80	1.20
50.0 or more	2%	3%

Inserts

Inserts are solid dosage forms that are inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum (see *Suppositories*). The drug substance in inserts is delivered for local or systemic action. Vaginal inserts are usually globular or oviform and weigh about 5 g each. Inserts intended to dissolve in vaginal secretions are usually made from water-soluble or water-miscible vehicles such as polyethylene glycol or glycerinated gelatin.

PREPARATION

For general considerations, see ⟨795⟩. Inserts vary considerably in their preparation. Inserts may be molded (using technology similar to that used to prepare lozenges, suppositories, or plastics), compressed from powders (as in tableting), or formulated as special applications of capsules (soft gelatin capsules and hard gelatin capsules have been employed for extemporaneously compounded preparations). Inserts may be formulated to melt at body temperature or disintegrate upon insertion. Design of the dosage form should take into consideration the fluid volume available at the insertion site and minimize the potential to cause local irritation. Most inserts are formulated to ensure retention at the site of administration.

Irrigations

(See *Solutions.*)

Liquids

As a dosage form, a liquid consists of a pure chemical in its liquid state. Examples include mineral oil, isoflurane, and ether. This dosage form term is not applied to solutions.

Lotions

(See *Emulsions.*)

Lozenges

Lozenges are solid oral dosage forms that are designed to dissolve or disintegrate slowly in the mouth. They contain one or more drug substances that are slowly liberated from the, typically, flavored and sweetened base. They are frequently intended to provide local action in the oral cavity or the throat but also include those intended for systemic absorption after dissolution. The typical therapeutic categories of drug substances delivered in lozenges are antiseptics, analgesics, decongestants, antitussives, and antibiotics. Molded lozenges are called cough drops or pastilles but these terms are not used in naming pharmacopeial articles. Lozenges prepared by compression or by stamping or cutting from a uniform bed of paste are sometimes known as troches (a term not used in naming pharmacopeial articles). Compressed or stamped lozenges are often produced in a circular shape.

Lozenges can be made using sugars such as sucrose and dextrose, or can provide the benefits of a sugar-free formulation that is usually based on sorbitol or mannitol. Polyethylene glycols and hypromellose are sometimes included to slow the rate of dissolution.

PREPARATION

Excipients used in molded lozenge manufacture include gelatin, fused sucrose, sorbitol, or another carbohydrate base.

Molded lozenges using a sucrose or sorbitol base containing drug substances such as phenol, dextromethorphan, fentanyl, and dyclonine hydrochloride and menthol are prepared by cooking the sugar (sucrose, corn syrup, and sorbitol) and water at about 150° to reduce the water content to less than 2%. The molten sugar solution is transferred to a cooling belt or cooling table, and medicaments, flavorings, and colorings are added and thoroughly mixed while cooling. Individual dosage units of the desired shape are formed by filling the molten mass into molds. These lozenges are quickly cooled in the molds to trap the base in the glassy state. Once formed, the lozenges are removed from the molds and packaged. Care is taken to avoid excessive moisture during storage to prevent crystallization of the sugar base.

Compressed lozenges are made using excipients that may include a filler, binder, sweetening agent, flavoring agent, and lubricant. Sugars such as sucrose, sorbitol, and mannitol are often included because they can act as a filler and binder as well as serve as sweetening agents. Approved FD&C and D&C dyes or lakes (dyes adsorbed onto insoluble aluminum hydroxide) may also be present.

The manufacturing of compressed lozenges is essentially the same as that for conventional tableting, with the exception that a tablet press capable of making larger tablets and exerting greater force to produce harder tablets may be required (see *Tablets*).

The paste used to produce lozenges manufactured by stamping or cutting contains a moistening agent, sucrose, and flavoring and sweetening agents. The homogenous paste is spread as a bed of uniform thickness, and the lozenges are cut or stamped from the bed and are allowed to dry. Some lozenges are prepared by forcing dampened powders under low pressure into mold cavities and then ejecting them onto suitable trays for drying at moderate temperatures.

Ointments

Ointments are semisolid preparations generally intended for external application to the skin or mucous membranes. Drug substances delivered in ointments are intended for local action or for systemic absorption. Ointments usually contain less than 20% water and volatiles, and more than 50% hydrocarbons, waxes, or polyols as the vehicle. Ointment bases recognized for use as vehicles fall into four general classes: hydrocarbon bases, absorption bases, water-removable bases, and water-soluble bases.

HYDROCARBON BASES

Also known as oleaginous ointment bases, hydrocarbon bases allow the incorporation of only small amounts of an aqueous component. Ointments prepared from hydrocarbon bases act as occlusive dressings and provide prolonged contact of the drug substance with the skin. They are difficult to remove and do not change physical characteristics upon aging.

ABSORPTION BASES

Allow the incorporation of aqueous solutions. Such bases include only anhydrous components (e.g., *Hydrophilic Petrolatum*) or water-in-oil emulsions (e.g., *Lanolin*). Absorption bases are also useful as emollients.

WATER-REMOVABLE BASES

Oil-in-water emulsions (e.g., *Hydrophilic Ointment*) are sometimes referred to as creams (see *Emulsions*). Water-removable bases may be readily washed from the skin or clothing with water, making them acceptable for cosmetic reasons. Other ad-

vantages of the water-removable bases are that they can be diluted with water and that they favor the absorption of serous discharges in dermatological conditions.

WATER-SOLUBLE BASES

Also known as greaseless ointment bases, they are formulated entirely from water-soluble constituents. *Polyethylene Glycol Ointment* is the only official preparation in this group. Water-soluble bases offer many of the advantages of the water-removable bases and, in addition, contain no water-insoluble substances such as petrolatum, anhydrous lanolin, or waxes. They are more correctly categorized as gels (see *Gels*).

The choice of an ointment base depends on the action desired, the characteristics of the incorporated drug substance, and the latter's bioavailability if systemic action is desired. The product's stability may require the use of a base that is less than ideal in meeting other quality attributes. Drug substances that hydrolyze rapidly, for example, are more stable in hydrocarbon bases than in bases that contain water.

PREPARATION

Ointments are typically prepared by either direct incorporation into a previously prepared ointment base or by fusion (heating during the preparation of the ointment). A levigating agent is often added to facilitate the incorporation of the medicament into the ointment base by the direct incorporation procedure. In the fusion method, the ingredients are heated. Homogenization is often necessary. The rate of cooling is an important manufacturing detail because rapid cooling can impart increased structure to the product of the fusion method.

General Chapters

Pastes

Pastes are semisolid preparations of stiff consistency and contain a high percentage (20%–50%) of finely dispersed solids. Pastes are intended for application to the skin, oral cavity, or mucous membranes. Pastes ordinarily do not flow at body temperature and thus can serve as occlusive, protective coatings. As a consequence, pastes are more often used for protective action than are ointments.

Fatty pastes that have a high proportion of hydrophilic solids appear less greasy and are more absorptive than ointments. They are used to absorb serous secretions and are often preferred for acute lesions that have a tendency toward crusting, vesiculation, or oozing.

Dental pastes are applied to the teeth. Other orally administered pastes may be indicated for adhesion to the mucous membrane for a local effect.

In veterinary medicine, pastes are typically administered orally and are intended for systemic delivery of drug substances. The paste is squeezed into the mouth of the animal, generally at the back of the tongue, or is spread inside the mouth.

Pellets

Pellets are dosage forms composed of small, solid particles of uniform shape sometimes called beads, although the use of the term "beads" as a dosage form is not preferred. Typically, pellets are nearly spherical but this is not required. Pellets may be administered by the oral (gastrointestinal) or by the injection route (see also *Implants*). Pellet formulations may provide several advantages, including physical separation for chemically or physically incompatible materials, extended release of the drug substance, or delayed release to protect an acid-labile drug substance from degradation in the stomach or to protect stomach tissues from irritation. Extended-release pellet formulations may be designed with the drug substance dispersed in a matrix, or the pellet may be coated with an appropriate polymer coating that modifies the drug-release characteristics. Alternatively, the pellet design may combine these two approaches. In the case of delayed-release formulations, the coating polymer is chosen to resist dissolution at the lower pH of the gastric environment but to dissolve in the higher pH intestinal environment. Injected or surgically administered pellet preparations (see *Implants*) are often used to provide continuous therapy for periods of months or years.

Pellet dosage forms may be designed as single or multiple entities. Often, implanted pellets will contain the desired drug substance content in one or several units. Oral pellets are typically contained within hard gelatin capsules for administration. Although there are no absolute requirements for size, the useful size range of pellets is governed by the practical constraints of the volume of commonly used capsules and the need to include sufficient numbers of pellets in each dose to ensure uniform dosing of the drug substance. As a result, many pellets used for oral administration fall within a size range of 710 µm to 2.5 mm. Pellet formulations are sometimes used to minimize variability associated with gastric retention of larger dosage forms.

Delayed-release pellet formulations and some extended-release formulations are prepared by applying a coating to the formulated particles. The coating must be applied as a continuous film over the entire surface of each particle. Because a small population of imperfectly coated particles may be unavoidable, oral pellets are designed to require the administration of a large number in a single dose to minimize any adverse influence of imperfectly coated pellets on drug delivery.

PREPARATION

The desired performance characteristics determine the manufacturing method chosen. In general, pellet dosage forms are manufactured by wet extrusion processes followed by spheronization, by wet or dry coating processes, or by compression. Manufacture of pellets by wet coating usually involves the application of successive coatings upon nonpareil seeds. This manufacturing process is frequently conducted in fluid-bed processing equipment. Dry powder coating or layering processes are often performed in specialized rotor granulation equipment. The extent of particle growth achievable in wet coating processes is generally more limited than the growth that can be obtained with dry powder layering techniques, but either method allows the formulator to develop and apply multiple layers of coatings to achieve the desired release profile. The manufacture of pellets by compression is largely restricted to the production of material for subcutaneous implantation. This method of manufacture provides the necessary control to ensure dose uniformity and is generally better suited to aseptic processing requirements.

Alternatively, microencapsulation techniques can be used to manufacture pellets. Coacervation coating techniques typically produce coated particles that are much smaller than those made by other techniques.

Pills

Pills are drug substance-containing small, spherical, solid bodies intended for oral administration. The pill dosage form has been largely replaced by compressed tablets and by capsules. Unlike tablets, pills are usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms, such as tablets and capsules.

PREPARATION

Excipients are selected on the basis of their ability to produce a mass that is firm and plastic. The drug substance is triturated with powdered excipients in serial dilutions to attain a uniform mixture. Liquid excipients that act to bind and provide plasticity to the mass are subsequently added to the dry materials. The mass is formed by kneading. The properties of firmness and plasticity are necessary to permit the mass to be worked and retain the shape produced. Cylindrical pill pipes are produced from portions of the mass. The pill pipe is cut into individual lengths corresponding to the intended pill size, and the pills are rolled to form the final shape. Pill-making machines can automate the preparation of the mass, production of pill piping, and the cutting and rolling of pills.

Plasters

A plaster is a semisolid substance for external application that is supplied on a support material. Plasters are applied for prolonged periods to provide protection, support, or occlusion (maceration). This term is not preferred and should not be used for new drug product titles. Plasters consist of an adhesive layer that may contain active substances. This layer is spread uniformly on an appropriate support that is usually made of a rubber base or synthetic resin. Unmedicated plasters are designed to provide protection or mechanical support to the site of application. Plasters are available in a range of sizes or cut to size to effectively provide prolonged contact to the site of application. They adhere firmly to the skin but can be peeled off the skin without causing injury.

Powders

Powders are defined as a single solid or a mixture of solids in a finely divided state. Powders used as pharmaceutical dosage forms may contain one or more drug substances and can be used as is or can be mixed with a suitable vehicle for administration. Powders are grouped according to the following terms: very coarse, coarse, moderately coarse, fine, and very fine (see *Powder Fineness* ⟨811⟩). Powders can be intended for internal or external use. Powders for external use are typically dusted onto the skin or applied to bandages or clothing. Powders for internal use can be applied to accessible mucous membranes with suitable applicators or are entrained in air streams for application to the nose or lungs.

The performance of powder dosage forms can be affected by the physical characteristics of the powder. For example, particle size can influence the dissolution rate of the particles and thus the bioavailability and/or effectiveness at the site of action. Externally applied powders should have a particle size of 150 µm or less (typically in the 50- to 100-µm range to prevent a gritty feel on the skin that could further irritate traumatized skin). The particle size of powders delivered to the lung or nose influences where the powder is deposited. Particle size can influence the mixing, segregation, and aggregation of the particles, which can affect the delivery and uniformity of the dosage form.

In veterinary medicine, a powder that needs to be reconstituted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term "concentrate" is no longer preferred.

INHALATION POWDERS AND NASAL POWDERS

Inhalation powders and nasal powders consist of an appropriately finely divided solid and a suitable container–closure delivery system. For additional information, see ⟨5⟩ and ⟨601⟩.

PREPARATION

Powder dosage forms can be produced by the combination of multiple components into a uniform blend. This preparation can also involve particle size reduction, a process referred to as comminution. Milling, spray drying, supercritical fluid, high-pressure homogenization, precipitation technologies, and porous microparticle fabrication techniques may be used to reduce the particle size of powders. As the particle size is decreased, the number of particles and the surface area increase, which can increase the dissolution rate and bioavailability, and/or the rate and extent of local action, of the drug substance.

Blending of powders may be accomplished by different techniques. Industrial processes may employ sifting or tumbling the powders in a rotating container. One of the most common tumble blenders is a V-blender, which is available in a variety of sizes suitable for small-scale and large-scale compounding and industrial production. Depending on the particle size of the drug substance, a random mixture of powders may be employed. Blending techniques for powders include those used in compounding pharmacy such as spatulation and trituration (see ⟨795⟩).

Powder flow can be influenced by both particle size and shape. Larger particles generally flow more freely than do fine particles. Powder flow is an important attribute that can affect the packaging or dispensing of a powder.

Soaps and Shampoos

Soaps and shampoos are solid or liquid preparations intended for topical application to the skin or scalp followed by subsequent rinsing with water. Soaps and shampoos are emulsions, suspensions, or surface-active compositions that readily form emulsions, micelles, or foams upon the addition of water followed by rubbing. Incorporation of drug substances in soaps and shampoos combines the cleansing/degreasing abilities of the vehicle and facilitates the topical application of the drug substance to affected areas, even large areas, of the body. The surface-active properties of the vehicle facilitate contact of the drug substance with the skin or scalp. Medicated soap and shampoo formulations frequently contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination.

PREPARATION

The preparation of medicated soaps and shampoos follows techniques frequently used for the preparation of emulsified systems. To ensure uniformity, the drug substance(s) must be added to the vehicle prior to congealing (in the case of soaps) followed by thorough mixing. If the medication is present as a suspension, the particle size must be controlled to promote uniform distribution of the drug substance and possibly optimize performance. Because soap manufacture frequently involves processing the ingredients at an elevated temperature, care must be exercised to avoid excessive degradation of the drug substance during processing.

Solutions

A solution is a preparation that contains one or more dissolved chemical substances in a suitable solvent or mixture of mutually miscible solvents. Because molecules of a drug substance in solution are uniformly dispersed, the use of solutions as dosage forms generally provides assurance of uniform dosage upon administration and good accuracy when the solution is diluted or otherwise mixed.

Substances in solutions are more susceptible to chemical instability than they are in the solid state and, dose-for-dose, are generally heavier and more bulky than solid dosage forms. These factors increase the cost of packaging and shipping relative to that of solid dosage forms. Solution dosage forms can be administered by injection, inhalation, and the mucosal, topical/dermal, and gastrointestinal routes. A solution administered by injection is officially titled "injection" (see ⟨1⟩).

Some solutions are designed to form a mass in situ. These solutions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the solution to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Solutions intended for oral administration usually contain flavorings and colorants to make the medication more attractive and palatable for the patient or consumer. When needed, they also may contain stabilizers to maintain chemical and physical stability and preservatives to prevent microbial growth.

Solutions are sometimes placed on devices such as swabs, cloths, or sponges, that aid application.

In veterinary medicine, a solution that needs to be diluted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term "concentrate" is no longer preferred.

Sprays

Spray preparations may deliver either accurately metered or nonmetered amounts of formulation.

A spray drug product is a dosage form that contains a drug substance in the liquid state as a solution or suspension and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

Depending on the design of the formulation and the valve system, the droplets generated may be intended for immediate inhalation through the mouth and deposition in the pulmonary tree, or for inhalation into the nose and deposition in the nasal cavity.

The mechanism for droplet generation and the intended use of the preparation distinguish various classes of sprays. A spray may be composed of a pump, container, actuator, valve, nozzle, or mouthpiece in addition to the formulation containing the drug(s), solvent(s), and any excipient(s). The design of each component plays a role for the appropriate performance of the drug product and in determining the critical characteristics of the droplet size distribution. Droplet and particle size distributions, delivered dose uniformity, plume geometry, and droplet velocity are critical parameters that influence the efficiency of drug delivery. When the preparation is supplied as a multidose container, the addition of a suitable antimicrobial preservative may be necessary. Spray formulations intended for local or systemic effect typically have an aqueous base and may contain excipients to control pH and viscosity. In addition, depending on the route of administration, the formulation may be isotonic. For additional information, see 〈5〉 and 〈601〉.

LABELING AND USE

Refer to CDER *Guidance for Industry: Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products—Chemistry, Manufacturing, and Controls Documentation.*

Strips

A strip is a dosage form or device in the shape of a long, narrow, thin, absorbant, solid material such as filter paper. Typically it is sterile and it may be impregnated with a compound or be gauged to allow measurements for diagnostic purposes, such as in measuring tear production. The term "strip" should not be used when another term such as "film" is more appropriate.

Suppositories

Suppositories are dosage forms adapted for application into the rectum. They melt, soften, or dissolve at body temperature. A suppository may have a local protectant or palliative effect, or may deliver a drug substance for systemic or local action.

Suppository bases typically include cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights, and fatty acid esters of polyethylene glycol. The suppository base can have a notable influence on the release of the drug substance(s). Although cocoa butter melts quickly at body temperature, it is immiscible with body fluids and this inhibits the diffusion of fat-soluble drug substances to the affected sites. Polyethylene glycol is a suitable base for some antiseptics. In cases when systemic action is desired, incorporating the ionized rather than the nonionized form of the drug substance may help maximize bioavailability. Although nonionized drug substances partition more readily out of water-miscible bases such as glycerinated gelatin and polyethylene glycol, the bases themselves tend to dissolve very slowly, which slows drug substance release. Cocoa butter and its substitutes (e.g., *Hard Fat*) perform better than other bases for allaying irritation in preparations intended for treating internal hemorrhoids. Suppositories for adults are tapered at one or both ends and usually weigh about 2 g each.

PREPARATION

Cocoa butter suppositories have cocoa butter as the base and can be made by incorporating the finely divided drug substance into the solid oil at room temperature and suitably shaping the resulting mass, or by working with the oil in the melted state and allowing the resulting suspension to cool in molds. A suitable quantity of hardening agents may be added to counteract the tendency of some drug substances (such as chloral hydrate and phenol) to soften the base. The finished suppository melts at body temperature.

A variety of vegetable oils, such as coconut or palm kernel, modified by esterification, hydrogenation, or fractionation, are used as cocoa butter substitutes to obtain products that display varying compositions and melting temperatures (e.g., *Hydrogenated Vegetable Oil* and *Hard Fat*). These products can be designed to reduce rancidity while incorporating desired characteristics such as narrow intervals between melting and solidification temperatures, and melting ranges to accommodate formulation and climatic conditions.

Drug substances can be incorporated into glycerinated gelatin bases by addition of the prescribed quantities to a vehicle consisting of about 70 parts of glycerin, 20 parts of gelatin, and 10 parts of water.

Several combinations of polyethylene glycols that have melting temperatures that are above body temperature are used as suppository bases. Because release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in preparation and storage than is the case for melting-type vehicles. However, high concentrations of higher molecular weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention.

Several nonionic surface-active agents closely related chemically to the polyethylene glycols can be used as suppository vehicles. Examples include polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene stearates. These surfactants are used alone or in combination with other suppository vehicles to yield a wide range of melting temperatures and consistencies. A notable advantage of such vehicles is their water dispersibility. However, care must be taken with the use of surfactants because they may either increase the rate of drug substance absorption or interact with the drug substance to reduce therapeutic activity.

Compounding suppositories using a suppository base typically involves melting the suppository base and dissolution or dispersion of the drug substance in the molten base (see ⟨795⟩). When compounding suppositories, the compounding professional prepares an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. In compounding suppositories, avoid caustic or irritating ingredients, carefully select a base that will allow the drug substance to provide the intended effect, and in order to minimize abrasion of the rectal membranes, reduce solid ingredients to the smallest reasonable particle size.

Suspensions

A suspension is a biphasic preparation consisting of solid particles dispersed throughout a liquid phase. Suspension dosage forms may be formulated for specific routes of administration such as oral, topical, inhalation, ophthalmic, otic, and injection. Some suspensions are prepared and ready for use, and others are prepared as solid mixtures intended for reconstitution with an appropriate vehicle just before use.

Inhalation suspensions (see ⟨5⟩), ophthalmic suspensions, injectable suspensions, and some otic suspensions are prepared in sterile form. Suspensions are generally not injected intravenously, epidurally, or intrathecally unless the product labeling clearly specifies these routes of administration.

Some liposomal drug products are referred to as suspensions because they can settle and require resuspension prior to administration (see ⟨1⟩).

Some suspensions are designed to form a mass in situ. These suspensions comprise polymer, drug substance, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the suspension to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the drug substance and extends the drug substance release for days or months.

Historically, the term "milk" was sometimes used for suspensions in aqueous vehicles intended for oral administration (e.g., *Milk of Magnesia*). The term "magma" is often used to describe suspensions of inorganic solids, such as clays in water, that display a tendency toward strong hydration and aggregation of the solid, giving rise to gel-like consistency and thixotropic rheological behavior (e.g., *Bentonite Magma*). In the past, the term "lotion" referred to both topical suspensions and topical emulsions. Now the term only refers to topical emulsions (see *Emulsions*).

Limited aqueous solubility of the drug substance(s) is the most common rationale for developing a suspension. Other potential advantages of an oral suspension include taste masking and improved patient compliance because of the more convenient dosage form. When compared to solutions, suspensions can have improved chemical stability. Ideally, a suspension should contain small uniform particles that are readily suspended and easily redispersed following settling. Unless the dispersed solid is colloidal, the particulate matter in a suspension will likely settle to the bottom of the container upon standing. Such sedimentation may lead to caking and solidification of the sediment and difficulty in redispersing the suspension upon agitation. To prevent such problems, manufacturers commonly add ingredients to increase viscosity and the gel state of the suspension or flocculation, including clays, surfactants, polyols, polymers, or sugars. Frequently, thixotropic vehicles are used to counter particle-settling tendencies, but these vehicles must not interfere with pouring or redispersal. Additionally, the density of the dispersed phase and continuous phase may be modified to further control settling rate. For topical suspensions, rapid drying upon application is desirable.

Temperature can influence the viscosity (and thus suspension properties and the ease of removing the dose from the bottle), and temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. When manufacturers conduct stability studies to establish product shelf life and storage conditions, they should cycle conditions (freeze/thaw) to investigate temperature effects.

Unless studies confirm that the formulation will not support microbial growth, suspension preparations packaged to provide multiple doses should contain suitable antimicrobial agents to protect against bacterial, yeast, and mold contamination (see ⟨51⟩) or other appropriate measures should be taken to avoid microbial contamination.

Suspensions for reconstitution are dry powder or granular mixtures that require the addition of water or a supplied formulated diluent before administration. This formulation approach is frequently used when the chemical or physical stability of the drug substance or suspension does not allow sufficient shelf life for a preformulated suspension. Typically, these suspensions are refrigerated after reconstitution to increase their shelf life. For this type of suspension, the powder blend is uniform and the powder readily disperses when reconstituted.

Injectable suspensions are generally intended for either subcutaneous or intramuscular routes of administration and should have a controlled particle size, typically in the range of 5 µm or smaller. The rationale for the development of injectable suspensions may include poor drug substance solubility, improved chemical stability, prolonged duration of action, and avoidance of first-pass metabolism. Care is needed in selecting the sterilization technique because it may affect product stability or alter the physical properties of the material.

In veterinary medicine, a suspension that needs to be diluted prior to administration has been called a concentrate (e.g., drug products administered via drinking water). Such use of the term "concentrate" is no longer preferred.

PREPARATION

Suspensions are prepared by adding suspending agents or other excipients and purified water or oil to solid drug substances and mixing to achieve uniformity. In the preparation of a suspension, the characteristics of both the dispersed phase and the dispersion medium should be considered. During development, manufacturers should define an appropriate particle size distribution for the suspended material to achieve the desired effectiveness and to minimize the likelihood of particle size changes during storage.

In some instances, the dispersed phase has an affinity for the vehicle and is readily wetted upon its addition. For some materials, the displacement of air from the solid surface is difficult, and the solid particles may clump together or float on top of the vehicle. In the latter case, a wetting agent may be used for certain types of suspensions to facilitate displacement of air from the powder surface. Surfactants, alcohol, glycerin, and other hydrophilic liquids can be used as wetting agents when an aqueous vehicle will be used as the dispersion phase. These agents function by displacing the air in the crevices of the particles and dispersing the particles. In the large-scale preparation of suspensions, wetting of the dispersed phase may be aided by the use of high-energy mixing equipment such as colloid mills or other rotor–stator mixing devices.

After the powder has been wetted, the dispersion medium (containing the soluble formulation components such as colorants, flavorings, and preservatives) is added in portions to the powder, and the mixture is thoroughly blended before subsequent additions of the vehicle. A portion of the vehicle is used to wash the mixing equipment free of suspended material, and this portion is used to bring the suspension to final volume and ensure that the suspension contains the desired concentration of solid matter. The final product may be passed through a colloid mill or other blender or mixing device to ensure uniformity.

Suspensions are resuspended before the dose is dispensed. Because of the viscosity of many suspension vehicles, air entrainment may occur during dosing. The formulation process allows evaluation of this possibility; adjustments in vehicle viscosity or the incorporation of low levels of antifoaming agents are common approaches to minimize air entrainment. Alternatively, specific instructions for resuspending the formulation may be provided to minimize air incorporation and ensure accurate dosing.

Systems

Systems are preparations of drug substance(s) in carrier devices, often containing adhesive backing, that are applied topically or inserted into body cavities. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use. The term "system" should not be used when another dosage form term is more appropriate (e.g., inserts and implants).

The notation of strength is either defined in terms of the amount of the drug substance released from the system over a specific period of time or as the drug concentration within the formulation (e.g., the percentage of the drug). Various routes of administration are possible, so the route must always be indicated in the compendial name when a specific location for application is essential for proper use (e.g., "intrauterine","ocular", or "periodontal" as the route of administration). For example, systems applied to the eye are called ocular systems. The route is named "transdermal" when, for example, systemic absorption of the drug substance may take place through the dermis without specifying the region of the body to which the system is applied.

The term "patch" has sometimes been used but is not preferred for use in drug product monograph nomenclature when referring to a system.

Intrauterine systems are intended for placement in the uterus. Release of the drug substance can be up to 5 years.

Ocular systems are intended for placement in the lower conjunctival fornix from which the drug diffuses through a membrane at a constant rate.

Periodontal systems are intended for placement in the pocket between the tooth and the gum. In some cases, periodontal systems may be formed in situ in the periodontal pocket and release the drug substance(s) for several weeks.

Transdermal systems (TDS) are placed onto intact skin to deliver the drug to the systemic circulation. They are designed for prolonged release (up to 7 days). Specific quality tests for TDSs are found in ⟨3⟩.

Tablets

Tablets are solid dosage forms in which the drug substance is generally blended with excipients and compressed into the final dosage. Tablets are the most widely used dosage form in the United States. Tablet presses use steel punches and dies to

prepare compacted tablets by the application of high pressures to powder blends or granulations. Tablets can be produced in a wide variety of sizes, shapes, and surface markings. Capsule-shaped tablets are commonly referred to as caplets, although the term is not preferred as a dosage form name. Specialized tablet presses may be used to produce tablets with multiple layers or with specially formulated core tablets placed in the interior of the final dosage form. These specialized tablet presentations can delay or extend the release of the drug substance(s) or physically separate incompatible drug substances. Tablets may be coated by a variety of techniques to provide taste masking, protection of photo-labile drug substance(s), extended or delayed release, or unique appearance (colors). When no deliberate effort has been made to modify the drug substance release rate, tablets are referred to as immediate-release.

BUCCAL TABLETS

Intended to be inserted in the buccal pouch, where the drug substance is absorbed directly through the oral mucosa. Few drug substances are readily absorbed in this way (examples are nitroglycerin and certain steroid hormones).

CHEWABLE TABLETS

Formulated and manufactured to produce a pleasant-tasting residue in the mouth and to facilitate swallowing. Hard chewable tablets are typically prepared by compaction, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and contain colors and flavors to enhance their appearance and taste. Soft chewable tablets are typically made by a molding or extrusion process, frequently with more than 10% water to help maintain a pliable, soft product. Hard chewable tablets in veterinary medicine often have flavor enhancers like brewer's yeast or meat/fish-based flavors.

Tablets for human use that include "Chewable" in the title must be chewed or crushed prior to swallowing to ensure reliable release of the drug substance(s) or to facilitate swallowing. If tablets are designed so that they may be chewed (but chewing is not required for drug substance release or ease of swallowing), the title should not include a reference to "chewable". In that case, the product may still be described as "chewable" in the ancillary labeling statement.

Tablets for veterinary use that are intended to be chewed will include "Chewable" in the title. However, it is understood that for veterinary products it is not possible to ensure that tablets are chewed prior to ingestion. Chewable tablets may be broken into pieces and fed to animals that normally swallow treats whole.

EFFERVESCENT TABLETS

Prepared by compaction and contain, in addition to the drug substance(s), mixtures of acids (e.g., citric acid or tartaric acid) and carbonates, and/or sodium bicarbonate. Upon contact with water, these formulations release carbon dioxide, producing the characteristic effervescent action.

HYPODERMIC TABLETS

Molded tablets made from completely and readily water-soluble ingredients; formerly intended for use in making preparations for hypodermic injection. They may be administered orally or sublingually when rapid drug substance availability is required.

MODIFIED-RELEASE TABLETS

There are two categories of modified-release tablet formulations recognized by USP:

Delayed-release tablets: Tablets are sometimes formulated with acid-resistant or enteric (also called gastro-resistant) coatings to protect acid-labile drug substances from the gastric environment or to prevent adverse events such as irritation.

Extended-release tablets: Extended-release tablets are formulated in such a manner as to make the drug substance available over an extended period of time following ingestion. Expressions such as "prolonged-release", "repeat-action", "controlled-release", and "sustained-release" have also been used to describe such dosage forms. However, the term "extended-release" is used for Pharmacopeial purposes. Requirements for dissolution (see ⟨711⟩) are typically specified in the individual monographs.

ORALLY DISINTEGRATING TABLETS

Orally disintegrating tablets are intended to disintegrate rapidly within the mouth to provide a dispersion before the patient swallows the resulting slurry where the drug substance is intended for gastrointestinal delivery and/or absorption. Some of these dosage forms have been formulated to facilitate rapid disintegration and are manufactured by conventional means or by using lyophilization or molding processes. Further details may be found in the CDER *Guidance for Industry: Orally Disintegrating Tablets.*

SUBLINGUAL TABLETS

Sublingual tablets are intended to be inserted beneath the tongue, where the drug substance is absorbed directly through the oral mucosa. As with *Buccal tablets*, few drug substances are extensively absorbed in this way, and much of the drug substance is swallowed and is available for gastrointestinal absorption.

TABLETS FOR ORAL SOLUTION

Before administration, tablets for oral solution are intended to be solubilized in a liquid diluent. In some cases, tablets for oral solution may also be chewed or swallowed.

TABLETS FOR ORAL SUSPENSION

Tablets for oral suspension are intended to be dispersed in a liquid before administration as a suspension. The dosage form is tablets for oral suspension when either the drug substance or the excipients do not dissolve when dispersed in a liquid. In some cases, tablets for oral suspension may also be chewed or swallowed.

TABLET TRITURATES

Small, usually cylindrical, molded or compacted tablets. Tablet triturates traditionally were used as dispensing tablets in order to provide a convenient, measured quantity of a potent drug substance for compounding purposes, but they are rarely used today.

PREPARATION

Most compacted (compressed) tablets consist of the drug substance(s) and a number of excipients. These excipients may include fillers (diluents), binders, disintegrating agents, lubricants, and glidants. Approved FD&C and D&C dyes or lakes, flavors, and sweetening agents may also be present.

Fillers or diluents are added when the quantity of drug substance(s) is too small or the properties of the drug substance do not allow satisfactory compaction in the absence of other ingredients. Binders impart adhesiveness to the powder blend and promote tablet formation and maintenance of drug substance uniformity in the tableting mixture. Disintegrating agents facilitate reduction of the tablet into small particles upon contact with water or biological fluids. Lubricants reduce friction during the compaction and ejection cycles. Glidants improve powder fluidity, powder handling properties, and tablet weight control. Colorants are often added to tablet formulations for aesthetic value or for product identification.

Tablets are prepared from formulations that have been processed by one of three general methods: wet granulation, dry granulation (roll compaction or slugging), and direct compression.

Wet granulation: Involves the mixing of dry powders with a granulating liquid to form a moist granular mass that is dried and sized prior to compression. It is particularly useful in achieving uniform blends of low-dose drug substances and facilitating the wetting and dissolution of poorly soluble, hydrophobic drug substances.

Dry granulations: Can be produced by passing powders between rollers at elevated pressure (roll compaction). Alternatively, dry granulation can also be carried out by the compaction of powders at high pressures on tablet presses, a process also known as slugging. In either case, the compacts are sized before compression. Dry granulation improves the flow and handling properties of the powder formulation without involving moisture in the processing.

Direct compression: Tablet processing involves dry blending of the drug substance(s) and excipients followed by compression. The simplest manufacturing technique, direct compression is acceptable only when the drug substance and excipients possess acceptable flow and compression properties without prior process steps.

Tablets may be coated to protect the ingredients from air, moisture, or light; to mask unpleasant tastes and odors; to improve tablet appearance; and to reduce dustiness. In addition, coating may be used to protect the drug substance from acidic pH values associated with gastric fluids or to control the rate of drug release in the gastrointestinal tract.

The most common coating in use today is a thin film coating composed of a polymer that is derived from cellulose. Sugar coating is an alternative, less common approach. Sugar-coated tablets have considerably thicker coatings that are primarily sucrose with a number of inorganic diluents. A variety of film-coating polymers are available and enable the development of specialized release profiles. These formulations are used to protect acid-labile drug substances from the acidic stomach environment as well as to prolong the release of the drug substance to reduce dosing frequency (see 〈711〉 or 〈701〉).

Tapes

A tape is a dosage form suitable for delivering drug substances to the skin. It consists of a drug substance(s) impregnated into a durable yet flexible woven fabric or extruded synthetic material that is coated with an adhesive agent. Typically the impregnated drug substance is present in the dry state. The adhesive layer is designed to hold the tape securely in place without

the aid of additional bandaging. Unlike transdermal systems, tapes are not designed to control the release rate of the drug substance. The term "tape" is not preferred and should not be used for new drug product titles.

The drug substance content of tapes is expressed as amount per surface area with respect to the tape surface exposed to the skin. The use of an occlusive dressing with the tape enhances the rate and extent of delivery of the drug substance to deeper layers of the skin and may result in greater systemic absorption of the drug substance.

GLOSSARY

This glossary provides definitions for terms in use in medicine and serves as a source of official names for official articles, except when the definition specifically states that the term is not to be used in drug product titles. Examples of general nomenclature forms for the more frequently encountered categories of dosage forms appear in ⟨1121⟩. In an attempt to be comprehensive, this glossary was compiled without the limits imposed by current preferred nomenclature conventions. To clearly identify/distinguish preferred from not preferred terms, entries indicate when a term is not preferred and generally direct the user to the current preferred term. When a term is described as an attribute of a dosage form generally it is intended to distinguish the term from those used for actual dosage form titles. While attribute terms are typically not used as the official name for the dosage form, when they are used they identify a specialized presentation or characteristic of the dosage form. For example, the attribute "chewable" may be used with the dosage form term "tablets" to identify a specific type of tablet that must be chewed prior to swallowing.

Aerosol: A dosage form consisting of a liquid or solid preparation packaged under pressure and intended for administration as a fine mist. The descriptive term "aerosol" also refers to the fine mist of small droplets or solid particles that are emitted from the product.

Aromatic water (not preferred; see *Solution*): A clear, saturated, aqueous solution of volatile oils or other aromatic or volatile substances.

Aural (Auricular) (not preferred; see *Otic*): For administration into, or by way of, the ear.

Bead (not preferred; see *Pellets*): A solid dosage form in the shape of a small sphere. In most products a unit dose consists of multiple beads.

Bolus (not preferred; see *Tablet*): A large tablet intended for administration to large animals. Occasionally, the term "bolus" is used to describe a method of administration.

Buccal: Administration directed toward the cheek, generally from within the mouth.

Caplet (not preferred; see *Tablet*): Tablet dosage form in the shape of a capsule.

Capsule: A solid dosage form in which the drug substance, with or without other ingredients, is filled into either a hard or soft shell or coated on the capsule shell. Most capsule shells are composed mainly of gelatin.

Chewable: Attribute of a solid dosage form that is intended to be chewed or crushed before swallowing.

Coating: Attribute (coated) of a solid dosage form that involves covering with an outer solid. The outer deposit is referred to as a coating or film. The term is used as an attribute when applied to solid oral dosage forms. Coatings are applied for functional or aesthetic purposes such as taste masking, stability, modifying release characteristics, product identification, and appearance.

Collodion (not preferred; see *Solution*): A preparation that is a solution dosage form composed of pyroxilin dissolved in a solvent mixture of alcohol and ether, and applied externally.

Colloidal dispersion: An attribute of a preparation or formulation in which particles of colloidal dimension (i.e., typically between 1 nm and 1 μm) are distributed uniformly throughout a liquid.

Concentrate (not a preferred term for human or veterinary drug products): The current use is for drug substances that are not intended for direct administration to humans or animals. The use in drug product nomenclature is being phased out (see ⟨1121⟩ and *Nomenclature Guidelines*[5]).

Conventional-release (not preferred; see *Immediate-release*): Descriptive term for a dosage form in which no deliberate effort has been made to modify the release rate of the drug substance. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification. This term is not used in drug product titles.

Cough drop: (not preferred; see *Lozenge*)

Cream: A semi-solid emulsion dosage form often containing more than 20% water and volatiles, and/or containing less than 50% hydrocarbons, waxes, or polyols as the vehicle for the drug substance. Creams are generally intended for external application to the skin or mucous membranes.

Delayed-release: A type of modified-release dosage form. A descriptive term for a dosage form deliberately modified to delay release of the drug substance for some period of time after initial administration. For example, release of the drug substance is prevented in the gastric environment but promoted in the intestinal environment; this term is synonymous with *Enteric-coated* or *Gastro-resistant*.

Dental: Descriptive term for a preparation that is applied to the teeth for localized action.

Dermal: A topical route of administration where the drug product is intended to reach or be applied to the dermis.

Dip (not preferred; see ***Immersion***)

Dispersible tablets (not preferred; see *Tablets*, *Tablets for oral suspension*, or *Tablets for oral solution*)

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[5] *Nomenclature Guidelines*, http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/2012-11-07_nom_guidelines.pdf.

Disintegrating tablets (not preferred; see *Tablets*, *Tablets for oral suspension*, or *Tablets for oral solution*): See also *Orally disintegrating*

Dosage form: A combination of drug substance(s) and/or excipient(s) in quantities and physical form designed to allow the accurate and efficient administration of the drug substance to the human or animal patient. The term is not used in drug product titles.

Dry powder inhaler: A device used to administer an inhalation powder in a finely divided state suitable for oral inhalation by the patient. This term is not used in drug product titles.

Effervescent: Attribute of an oral dosage form, frequently tablets or granules, containing ingredients that, when in contact with water, rapidly release carbon dioxide. The dosage form is dissolved or dispersed in water to initiate the effervescence prior to ingestion.

Elixir (not preferred; see *Solution*): A preparation that typically is a clear, flavored, sweetened hydroalcoholic solution intended for oral use. The term should not be used for new drug products in *USP–NF* but is commonly encountered in compounding pharmacy practice.

Emollient: Attribute of a cream or ointment indicating an increase in the moisture content of the skin following application of bland, fatty, or oleaginous substances. This term should not be used in drug product titles.

Emulsion: A dosage form consisting of a two-phase system composed of at least two immiscible liquids, one of which is dispersed as droplets (internal or dispersed phase) within the other liquid (external or continuous phase), generally stabilized with one or more emulsifying agents. Emulsion is not used as a dosage form term if a more specific term is applicable (e.g., *Cream*, *Lotion*, or *Ointment*).

Enteric-coated (not preferred; see *Delayed-release*): Descriptive term for a solid dosage form in which a polymer coating has been applied to prevent the release of the drug substance in the gastric environment.

Excipient: An ingredient of a dosage form other than a drug substance. This term is not used in drug product titles. The term "excipient" is synonymous with inactive ingredient.

Extended-release: Descriptive term for a dosage form that is deliberately modified to protract the release rate of the drug substance compared to that observed for an immediate-release dosage form. The term is synonymous with prolonged- or sustained-release. Many extended-release dosage forms have a pattern of release that begins with a "burst effect" that mimics an immediate release followed by a slower release of the remaining drug substance in the dosage form.

Film: A term used to describe a thin sheet of material, usually composed of a polymer. Films are used in various routes of administration including as a means of oral administration of material in a rapidly dissolving form.

Foam: A dosage form containing gas bubbles dispersed in a liquid. Medicated foams have a semisolid consistency and can be formulated to quickly break down into a liquid or to remain as foam to ensure prolonged contact.

Gas: One of the states of matter having no definite shape or volume and occupying the entire container when confined.

Gastro-resistant (not preferred; see *Delayed-release*): Descriptive term for a solid dosage form in which a polymer coating has been applied to prevent the release in the gastric environment.

Gel: A dosage form that is a semisolid dispersion of small particles or a solution of large molecules interpenetrated by a solution containing a gelling agent to provide stiffness.

Granules: A dosage form composed of dry aggregates of powder particles that may contain one or more drug substances, with or without other ingredients. They may be swallowed as such, dispersed in food, or dissolved in water. Granules are frequently compacted into tablets or filled into capsules, with or without additional ingredients. More commonly, granules are reconstituted as suspensions.

Gum: A dosage form in which the base consists of a pliable material that, when chewed, releases the drug substance into the oral cavity.

Hard-shell capsule (not preferred; see *Capsules*): A type of capsule in which one or more drug substances, with or without other ingredients, are filled into a two-piece shell. Most hard-shell capsules are composed mainly of gelatin and are fabricated prior to the filling operation.

Immediate-release: Descriptive term for a dosage form in which no deliberate effort has been made to modify the drug substance release rate. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification. This term is not used in drug product names.

Immersion: A veterinary route of administration via partial or complete submersion in a specified environment such as liquid or air.

Implant: A dosage form that is a solid or semisolid material containing the drug substance that is inserted into the body. The insertion process is invasive, and the material is intended to reside at the site for a period consistent with the design release kinetics or profile of the drug substance(s).

Inhalation (by inhalation): A route of administration for aerosols characterized by dispersion of the drug substance into the airways during inspiration.

Injection (by injection): A route of administration of a liquid or semisolid deposited into a body cavity, fluid, or tissue by use of a needle.

Injection: Liquid preparations that may contain drug substances and/or excipients or solutions thereof. The term "for injection" indicates dry solids that, upon the addition of a suitable vehicle, yield solutions conforming in all respects to the requirements for injections.

Injectable emulsion: Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.

Injectable suspension: Liquid preparations of solids suspended in a liquid medium. The term "for injectable suspension" indicates dry solids that, upon the addition of a suitable vehicle, yield preparations conforming in all respects to the requirements for injectable suspensions.

Extended-release injectable suspension: Liquid preparations of solids suspended in a suitable vehicle and formulated to allow the drug substance to be available over an extended period of time. The term "for extended-release injectable suspension" indicates dry solids that, upon the addition of a suitable vehicle, yield a preparation that conforms in all respects to the requirements for extended-release injectable suspensions.

Insert: A solid dosage form that is inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum. It should be noted that a suppository is intended for application into the rectum and is not classified as an insert (see *Suppository*).

Intraocular: A route of administration to deliver a sterile preparation within the eye.

Irrigation: A sterile solution or liquid intended to bathe or flush open wounds or body cavities.

Jelly (not preferred; see *Gel*): A semisolid dispersion of small particles or a solution of large organic molecules interpenetrated by a solution containing a gelling agent to promote stiffness.

Liposomes: Attribute for preparations of amphiphilic lipids that have low water solubility (see ⟨1⟩).

Liquid: A dosage form consisting of a pure chemical in its liquid state. This dosage form term should not be applied to solutions. The term is not used in drug product titles. When "liquid" is used as a descriptive term, it indicates a material that is pourable and conforms to its container at room temperature.

Lotion: An emulsion liquid dosage form applied to the outer surface of the body. Historically, this term was applied to topical suspensions and topical emulsions. The current definition of a lotion is restricted to an emulsion.

Lozenge: A solid dosage form intended to disintegrate or dissolve slowly in the mouth.

Modified-release: A descriptive term for a dosage form with a drug substance release pattern that has been deliberately changed from that observed for the immediate-release dosage form of the same drug substance. The two types of modified-release are extended-release and delayed-release. The term "modified-release" is not used in drug product titles.

Molded tablet: A tablet that has been formed by dampening the ingredients and pressing into a mold, then removing and drying the resulting solid mass. This term is not used in drug product titles.

Mouthwash (not preferred; see *Rinse*): Term applied to a solution preparation used to rinse the oral cavity.

Nasal: Route of administration (mucosal) characterized by administration to the nose or by way of the nose for local or systemic effect.

Ocular (not preferred; see *Intraocular*): Route of administration indicating deposition of the drug substance within the eye.

Ointment: A semisolid dosage form, usually containing less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. This dosage form generally is for external application to the skin or mucous membranes.

Ophthalmic: A route of administration characterized by application of a sterile preparation to the external parts of the eye.

Oral: Route of administration characterized by application to the mouth or delivery to the gastrointestinal tract through the mouth.

Orally disintegrating: A descriptive term for a solid oral dosage form that disintegrates rapidly in the mouth prior to swallowing. The drug substance is intended for gastrointestinal delivery and/or absorption. See also CDER *Guidance for Industry, Orally Disintegrating Tablets*.

Orodispersible: (not preferred; see *Orally disintegrating*)

Oro-pharyngeal: A route of administration characterized by deposition of a preparation into the oral cavity and/or pharyngeal region to exert a local or systemic effect.

Otic: A route of administration characterized by deposition of a preparation into, or by way of, the ear. Sometimes referred to as *Aural* (*Aural* not preferred).

Parenteral: General route of administration which is characterized by injection through the skin or other external boundary tissue or implantation within the body. Specific parenteral routes include intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intraocular (see ⟨1⟩).

Paste: A semisolid dosage form containing a high percentage (20%–50%) of finely dispersed solids with a stiff consistency. This dosage form is intended for application to the skin, oral cavity, or mucous membranes.

Pastille: (not preferred; see *Lozenge*)

Patch (not preferred; see *System*): Frequently incorrectly used to describe a *System*.

Pellet: A small solid dosage form of uniform, often spherical, shape intended for direct administration as a pellet. Spherical pellets are sometimes referred to as *Beads*. Pellets intended as implants must be sterile. The use of the term "pellet" for implantable dosage forms is no longer preferred (see *Implants*).

Periodontal: Descriptive term for a preparation that is applied around a tooth for localized action.

Pill: A solid, spherical dosage form usually prepared by a wet massing, piping, and molding technique. This term is frequently incorrectly used as a general term to describe solid oral dosage forms such as tablets or capsules.

Plaster (not preferred): A dosage form containing a semisolid composition supplied on a support material for external application. Plasters are applied for prolonged periods of time to provide protection, support, or occlusion (for macerating action).

Powder: A dosage form composed of a solid or mixture of solids reduced to a finely divided state and intended for internal or external use.

Powder, inhalation: A powder containing a drug substance for oral inhalation. The powder is used with a device that aerosolizes and delivers an accurately metered amount.

Premix: (not preferred; see *Type A Medicated Articles* and *Type B Medicated Articles* in *Animal Drugs for Use in Animal Feeds* 〈1152〉)

Prolonged-release: (not preferred; see *Extended-release*)

Rectal: A route of administration characterized by deposition into the rectum to provide local or systemic effect.

Rinse (see *Solution*): A liquid preparation used to cleanse by flushing. A rinse is used to swish in the mouth and then expectorated. The nonpreferred term "mouthwash" has sometimes been used for "rinse".

Semisolid: Attribute of a material that exhibits plastic flow behavior. A semisolid material is not pourable, does not readily conform to its container at room temperature, and does not flow at low shear stress. This term is not used in drug product titles.

Shampoo: A solution, emulsion, or suspension dosage form used to clean the hair and scalp. May contain a drug substance intended for topical application to the scalp.

Soap: The alkali salt(s) of a fatty acid or mixture of fatty acids used to cleanse the skin. Soaps used as dosage forms may contain a drug substance intended for topical application to the skin. Soaps have also been used as liniments and enemas.

Soft gel capsule (not preferred; see *Capsule*): A specific capsule type characterized by increased levels of plasticizers producing a more pliable and thicker-walled material than hard gelatin capsules. Soft gel capsules are further distinguished because they are single-piece sealed dosages. Frequently used for delivering liquid compositions.

Soluble tablets: (not preferred; see *Tablets* and *Tablets for oral solution*)

Solution: A clear, homogeneous liquid dosage form that contains one or more chemical substances dissolved in a solvent or mixture of mutually miscible solvents.

Spirit (not preferred; see *Solution*): A liquid dosage form composed of an alcoholic or hydroalcoholic solution of volatile substances.

Spot on (pour on): A method of delivering liquid veterinary drug products by administering them onto the animal's skin, usually between the shoulder blades (spot on) or down the back (pour on).

Spray: A spray is a dosage form that contains drug substance(s) in the liquid state, either as a solution or as a suspension, and is intended for administration as a mist. Sprays are distinguished from aerosols in that spray containers are not pressurized. Most of the sprays are generated by manually squeezing a flexible container or actuation of a pump that generates the mist by discharging the contents through a nozzle.

As an attribute, spray describes the generation of droplets of a liquid or solution to facilitate application to the intended area.

Stent, drug-eluting: A specialized form of implant used for extended local delivery of the drug substance to the immediate location of stent placement.

Strip (only used for diagnostic products, otherwise not preferred; see ***Film***): A dosage form or device in the shape of a long, narrow, thin, absorbent, solid material such as filter paper.

Sublingual: A route of administration characterized by placement underneath the tongue and for release of the drug substance for absorption in that region.

Suppository: A solid dosage form in which one or more drug substances are dispersed in a suitable base and molded or otherwise formed into a suitable shape for insertion into the rectum to provide local or systemic effect.

Suspension: A liquid dosage form that consists of solid particles dispersed throughout a liquid phase.

Syrup (not preferred; see *Solution*): A solution containing high concentrations of sucrose or other sugars. This term is commonly used in compounding pharmacy.

System: A preparation of drug substance(s) in a carrier device that is applied topically or inserted into a body cavity. The drug substance is designed to be released in a controlled manner over a specified period of time or the drug substance is released based on its concentration in the formulation. Unless otherwise stated in the labeling, the carrier device is removed after use.

Tablet: A solid dosage form prepared from powders or granules by compaction.

Tablets for oral solution: Tablets that are intended to be dispersed in a liquid before administration. When dispersed in the liquid, a solution results.

Tablets for oral suspension: Tablets that are intended to be dispersed in a liquid before administration. When dispersed in the liquid, a suspension results.

Tape (not preferred): A dosage form or device composed of a woven fabric or synthetic material onto which a drug substance is placed, usually with an adhesive on one or both sides to facilitate topical application. The rate of release of the drug substance is not controlled.

Tincture (not preferred; see *Solution*): An alcoholic or hydroalcoholic solution prepared from vegetable materials or from chemical substances.

Topical: A route of administration characterized by application to the outer surface of the body.

Transdermal: A route of administration through the dermal layer of the skin to the systemic circulation.

Troche (not preferred; see *Lozenge*): A solid dosage form intended to disintegrate or dissolve slowly in the mouth and usually prepared by compaction in a manner similar to that used for tablets.

Urethral: A route of administration characterized by deposition into the urethra.

Vaginal: A route of administration characterized by deposition into the vagina.

Vehicle: A term commonly encountered in compounding pharmacy that refers to a component for internal or external use that is used as a carrier or diluent in which liquids, semisolids, or solids are dissolved or suspended. Examples include water, syrups, elixirs, oleaginous liquids, solid and semisolid carriers, and proprietary products (see *Excipient*). This term is not used in drug product titles.

Veterinary: Descriptive term for dosage forms intended for nonhuman use.

〈1152〉 ANIMAL DRUGS FOR USE IN ANIMAL FEEDS

PURPOSE

This chapter provides general descriptions of, and definitions for, animal drugs and drug products delivered in animal feeds. It discusses general principles involved in the manufacture, packaging, and labeling of these drugs and drug products.

SCOPE

Medicated articles and feeds that are used to deliver animal drug(s) via the food given to animals are discussed in this general information chapter. Drugs approved for further manufacture into medicated animal feeds are not dosage form drugs. Dosage form drugs administered with feeds are not medicated articles or feeds. Drug dosage forms are listed in *USP* general chapter *Pharmaceutical Dosage Forms* 〈1151〉.

Animal drugs approved for further manufacture into medicated animal feeds may be in either dry or liquid form. They are sometimes referred to as premixes. The term "premix" is no longer used for animal drugs for use in animal feeds but is still used in some older drug monographs. Animal drugs in feeds are regulated as Type A medicated articles and Type B and Type C medicated feeds.

Type A Medicated Articles

Type A medicated articles [21 CFR 558.3(b)(2)] are concentrated forms of animal drugs intended solely for further manufacture of other approved Type A medicated articles or Type B or C medicated feeds. This means that Type A medicated articles cannot be fed directly to animals. They consist of one or more animal drug(s) with or without a carrier (e.g., calcium carbonate, rice hull, corn, gluten) and with or without other inactive ingredients. They can be prepared in dry or liquid form. They are sold to feed mills or livestock producers and are intended to be further diluted by mixing into feed before consumption by animals.

Type B Medicated Feeds

Type B medicated feeds [21 CFR 558.3(b)(3)] are intermediate medicated feeds for animals. They are manufactured from either Type A medicated article(s) or other Type B medicated feed(s) by dilution with non-medicated feed ingredients. In addition to the animal drug(s), Type B medicated feeds contain a substantial quantity of nutrients that comprise NLT 25% of the total feed weight. They can be prepared in dry or liquid form. Similar to Type A medicated articles, Type B medicated feeds are intended only for further dilution by mixing into feed, and they are not approved for feeding to animals.

Type C Medicated Feeds

Type C medicated feeds [21 CFR 558.3(b)(4)] are intended to be fed directly to animals. They are manufactured from Type A medicated articles, Type B medicated feeds, or other Type C medicated feeds diluted with non-medicated feed ingredients. Type C medicated feeds may be prepared in dry or liquid form. Type C medicated feeds can be either fed as the complete feed for the animals, top-dressed onto the animals' normal daily rations, or offered "free-choice" (21 CFR 510.455). Type C medicated feeds approved to be offered free-choice are not intended to be fully consumed in a single feeding or to constitute the entire diet of the animals.

PREPARATION

Type A medicated articles in dry form are typically produced by blending the drug substance(s) with carriers and other excipients to promote uniform mixing when subsequently added to the animal feed. The drug substance(s) may first be mixed with an excipient (e.g., starch or sodium aluminosilicate) that has a similar particle size and can help distribute the drug substance(s) uniformly throughout the final mixture. This pre-blend may then be mixed with bulk excipients (e.g., calcium carbonate or soybean hulls). The product may be granulated and/or oil (e.g., mineral oil, soybean oil) may be added to aid uniform distribution, to prevent particle segregation during shipping, and/or to minimize formation of airborne drug substance particles during production of another Type A medicated article or Types B or C medicated feeds.

Type A medicated articles in liquid form are produced by mixing the drug substance(s) with a suitable solvent (e.g., water or propylene glycol). The drug substance(s) is usually dissolved to produce a solution, but suspension products could be produced also.

Types B or C medicated feeds are typically manufactured at feed mills or on-farm by livestock producers. To manufacture Types B or C medicated feeds in dry form, Type A medicated articles are added to the feeds during the mixing process. Liquid Type A medicated articles may be sprayed in at set rates, and dry Type A medicated articles are added using methods that facilitate uniform distribution in the feeds. Types B and C medicated feeds in dry form may be further processed by heating, steaming, and extruding into pellets. The pellets may be rolled or broken up to create crumbles. Types B and C medicated feeds may also be prepared in liquid form. Liquid feeds are typically molasses based and contain an animal drug(s) dissolved or suspended in the liquid matrix. The liquid feed may need to be recirculated or agitated on a routine basis to maintain a uniform distribution of the drug(s).

A Type B medicated feed may also be prepared by diluting another Type B medicated feed. A Type C medicated feed may also be prepared by diluting a Type B medicated feed or another Type C medicated feed.

LABELING AND PACKAGING

Labeling for Type A medicated articles and Types B and C medicated feeds provides all information necessary for their safe and effective use. The label for a Type A medicated article includes mixing directions for the manufacture of medicated feeds from the Type A medicated article and feeding directions for the Type C medicated feeds. The label for a Type B medicated feed provides mixing directions for the manufacture of medicated animal feeds. Labels for both Type A medicated articles and Type B medicated feeds indicate that they are not to be fed directly to animals. The label for Type C medicated feeds includes directions for feeding.

Type A medicated articles are packaged in bags (e.g., paper with polyethylene liners) for dry products or in appropriate containers (e.g., plastic) for liquids. Typical sizes are 50-lb bags or several-gallon containers. Dry Types B and C medicated feeds may be packaged in bags for storage and delivery, or they may be shipped in bulk form for storage or immediate use. Free-choice Type C medicated feeds may be packaged in bags (e.g., loose minerals), wrapped in film (e.g., compressed blocks), or packaged in tubs (e.g., molded blocks). Liquid Types B and C medicated feeds are stored and shipped in bulk in tanks.

NOMENCLATURE

The drug product's established (non-proprietary) name consists of the drug substance (active moiety) and an appropriate type of medicated article or feed. The following nomenclature options are available to indicate types of medicated articles or feeds as part of the product established name:

[Drug] Type A medicated article
[Drug] Type A liquid medicated article
[Drug] Type B medicated feed
[Drug] Type B liquid medicated feed
[Drug] Type C medicated feed
[Drug] Type C liquid medicated feed
[Drug] Type C free-choice medicated feed
[Drug] Type C liquid free-choice medicated feed
[Drug] Type C top-dress medicated feed

⟨1160⟩ PHARMACEUTICAL CALCULATIONS IN PHARMACY PRACTICE

Table of Contents

1. *INTRODUCTION*
2. *CALCULATING AMOUNTS OF ACTIVE INGREDIENTS*

3. *DOSAGE CALCULATIONS*
4. *USE OF POTENCY UNITS*
5. *VOLUME AND WEIGHT SUMS*
6. *DENSITY AND SPECIFIC GRAVITY*
7. *MILLIEQUIVALENTS AND MILLIMOLES*
8. *CONCENTRATION EXPRESSIONS (with Table 1)*
9. *ALCOHOL*
10. *ALLIGATION ALTERNATE AND ALGEBRA METHODS*
11. *ALIQUOT CALCULATIONS*
12. *POWDER VOLUME CALCULATIONS*
13. *INTRAVENOUS FLOW OR INFUSION RATES*
14. *ISOOSMOTIC SOLUTIONS*
15. *pH AND BUFFER CALCULATIONS*
16. *TEMPERATURE*
17. *ENDOTOXINS*
18. *STABILITY AND EXPIRATION DATE CALCULATIONS*
19. *APPENDIX 1: LOGARITHMS (with Table 9 and Table 10)*

1. INTRODUCTION

The purpose of this general chapter is to provide general information to assist pharmacists and support personnel in performing the necessary calculations for compounding and dispensing medications. This general chapter is not inclusive of all the information necessary for performing pharmaceutical calculations. For additional information regarding pharmaceutical calculations, consult a pharmaceutical calculations textbook. For additional information on pharmaceutical compounding and drug stability, see: *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, and *Packaging and Storage Requirements* ⟨659⟩; *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩ and *Stability Considerations in Dispensing Practice* ⟨1191⟩.

Correct pharmaceutical calculations can be accomplished by using proper conversions from one measurement system to another and properly placing decimal points (or commas, in countries where it is customary to use these in the place of decimal points), by understanding the arithmetical concepts, and by paying close attention to the details of the calculations. Before proceeding with any calculation, pharmacists should do the following: (a) read the entire formula or prescription carefully; (b) determine the materials that are needed; and then (c) select the appropriate methods of preparation and the appropriate calculations.

Logical methods that require as few steps as possible should be selected to ensure that calculations are done accurately and correctly. A pharmacist should double-check each calculation or have someone else double-check, e.g., a technician, if another pharmacist is not available, before proceeding with compounding the preparation. One expedient method of double-checking is estimation, which consists of convenient rounding (e.g., 0.012 to 0.01, 0.44 to 0.5, 18.3 to 20, and 476 to 500) to approximate the magnitude of answers.

2. CALCULATING AMOUNTS OF ACTIVE INGREDIENTS

The pharmacist must be able to calculate the amount or concentration of drug substances in each unit or dosage portion of a compounded preparation at the time it is prepared and again at the time it is dispensed. Pharmacists must perform calculations and measurements to obtain, theoretically, 100% of the amount of each ingredient in compounded formulations. Calculations must account for the active ingredient, or active moiety, and water content of drug substances, which includes that in the chemical formulas of hydrates. Official drug substances and added substances must meet the requirements in general chapter *Loss on Drying* ⟨731⟩, which must be included in the calculations of amounts and concentrations of ingredients. The pharmacist should consider the effect of ambient humidity on the gain or loss of water from drugs and added substances in containers subjected to intermittent opening over prolonged storage. Each container should be opened for the shortest duration necessary and then closed tightly immediately after use.

The nature of the drug substance to be weighed and used in compounding a prescription must be known. If the substance is a hydrate, its anhydrous equivalent weight may need to be calculated. On the other hand, if there is adsorbed moisture present that is either specified on a Certificate of Analysis (CoA) or that is determined in the pharmacy immediately before the drug substance is used in the preparation (see chapter ⟨731⟩), this information must be used when calculating the amount of drug substance that is to be weighed to determine the exact amount of anhydrous drug substance required.

There are cases in which the required amount of a dose is specified in terms of a cation (e.g., Li^+), an anion (e.g., F^-), or a molecule (e.g., theophylline in aminophylline). In these instances, the drug substance weighed is a salt or complex, a portion of which represents the pharmacologically active moiety. Thus, the exact amount of such substances weighed must be calculated on the basis of the required quantity of the pharmacological moiety.

The following formula may be used to calculate the theoretical weight of an ingredient in a compounded preparation:

$$W = AB/CD$$

W = actual weighed amount
A = prescribed or pharmacist-determined weight of the active or functional moiety of drug or added substance
B = molecular weight (MW) of the ingredient, including waters of hydration for hydrous ingredients
C = MW of the active or functional moiety of a drug or added substance that is provided in the MW of the weighed ingredient
D = the fraction of dry weight when the percent by weight of adsorbed moisture content is known from the loss on drying procedure (see chapter ⟨731⟩) or from the CoA. The CoA should be lot specific.

2.1 Active Ingredients

2.1.1 CALCULATING DRUGS DOSED AS SALT FORM AND HYDRATE

Examples—Drugs dosed as salt form and hydrate

1. Drugs dosed as salt form and hydrate

Triturate morphine sulfate and lactose to obtain 10 g in which there are 30 mg of morphine sulfate for each 200 mg of the morphine–lactose mixture. [NOTE—Morphine is dosed as the morphine sulfate, which is the pentahydrate.]

W = weight of morphine sulfate (g)
A = weight of morphine sulfate pentahydrate in the prescription, 1.5 g
B = MW of morphine sulfate pentahydrate, 759 g/mol
C = MW of morphine sulfate pentahydrate, 759 g/mol
D = 1.0

To solve the equation:

$$W = (1.5 \text{ g} \times 759 \text{ g/mol})/(759 \text{ g/mol} \times 1) = 1.5 \text{ g of morphine sulfate pentahydrate}$$

2. Active drug moiety and correction for moisture

Accurately weigh an amount of aminophylline to obtain 250 mg of anhydrous theophylline. [NOTE—In this example, the powdered aminophylline dihydrate weighed contains 0.4% w/w absorbed moisture as stated in the CoA received by the pharmacy.]

$$W = AB/CD$$

W = weight of aminophylline dihydrate (mg)
A = weight of anhydrous theophylline, 250 mg
B = MW of aminophylline dihydrate, 456 g/mol
C = MW of anhydrous theophylline, 360 g/mol
D = 0.996

[NOTE—One mol of aminophylline contains 2 mol of theophylline. Theophylline has a MW of 180.]

To solve the equation:

$$W = (250 \text{ mg} \times 456 \text{ g/mol})/(360 \text{ g/mol} \times 0.996) = 318 \text{ mg of aminophylline dihydrate}$$

2.2 Hydrates, Salts, and Esters

Frequently, for stability or other reasons such as taste or solubility, the base form of a drug is administered in another form such as a salt or an ester. This altered form of the drug usually has a different MW, and at times it may be useful to determine the amount of the base form of the drug in the altered form.

2.2.1 CALCULATING HYDRATES, SALTS, AND ESTERS

Examples—Hydrates, salts, and esters

1. Hydrates

If a prescription for 100 g of lidocaine hydrochloride 2% gel is to be made, 2 g of anhydrous lidocaine hydrochloride could be used, or the equivalent amount of lidocaine hydrochloride monohydrate could be calculated as follows:

W = weight of lidocaine hydrochloride monohydrate (g)
A = weight of anhydrous lidocaine hydrochloride in the prescription, 2 g
B = MW of lidocaine hydrochloride monohydrate, 288.81 g/mol
C = MW of anhydrous lidocaine hydrochloride, 270.80 g/mol
D = 1.0

To solve the equation:

$$W = (2 \text{ g} \times 288.81 \text{ g/mol})/(270.80 \text{ g/mol} \times 1) = 2.133 \text{ g of lidocaine hydrochloride monohydrate}$$

2. Salts

A prescription calls for 10 mL of a fentanyl topical gel at a concentration 50 µg fentanyl/0.1 mL prepared from fentanyl citrate. The amount of fentanyl citrate required for the preparation could be calculated as follows:

Amount of fentanyl needed for the preparation:

$$(50\ \mu g\ \text{fentanyl}/0.1\ mL) \times 10\ mL = 5000\ \mu g\ \text{of fentanyl}$$

W = weight of fentanyl citrate in the prescription (µg)
A = weight of fentanyl in the prescription, 5000 µg
B = MW of fentanyl citrate, 528.59 g/mol
C = MW of fentanyl, 336.47 g/mol
D = 1.0

To solve the equation:

$$W = (5000\ \mu g \times 528.59\ g/mol)/(336.47\ g/mol \times 1) = 7855\ \mu g\ \text{of fentanyl citrate}$$

3. Esters

The amount of cefuroxime axetil contained in a single 250-mg cefuroxime tablet can be calculated as follows:

W = weight of cefuroxime axetil in tablet (mg)
A = weight of cefuroxime in the prescription, 250 mg
B = MW of cefuroxime axetil, 510.47 mg/mmol
C = MW of cefuroxime, 424.39 mg/mmol
D = 1.0

To solve the equation:

$$W = (250\ mg \times 510.47\ g/mol)/(424.39\ g/mol \times 1) = 300\ mg\ \text{of cefuroxime axetil}$$

3. DOSAGE CALCULATIONS

3.1 Dosing by Weight

Doses are frequently expressed as mg of drug per kg of body weight per a dosing interval.

3.1.1 CALCULATING DOSING BY WEIGHT

Example—Dosing by weight

A physician orders azithromycin for oral suspension at a dose of 15 mg/kg/day, divided every 12 h, for a child that weighs 36 lb. Calculate the volume of oral suspension, in mL, that should be administered for each dose of a 200-mg/5-mL suspension as follows:

(a) Calculate the child's weight in kg:

$$36\ lb \times kg/2.2\ lb = 16.4\ kg$$

(b) Multiply the weight, in kg, by the dosing rate:

$$16.4\ kg \times 15\ mg/kg/day = 246\ mg/day$$

(c) Divide the total daily dose by the number of doses/day:

$$246\ mg/2\ doses = 123\ mg/dose$$

(d) Calculate the volume of each dose using ratio and proportion:

$$(123\ mg/dose)/(200\ mg/5\ mL) = 3.1\ mL/dose$$

Some calculations may also be completed using dimensional units analysis (DUA). The DUA should begin at the left end with a factor containing the numerator answer units. All units other than those in the answer should cancel. If using DUA, the preceding equation would be as follows:

$$36\text{-lb child} \times \frac{kg}{2.2\ lb} \times \frac{15\ mg}{kg \cdot day} \times \frac{5\ mL}{200\ mg} \times \frac{day}{2\ doses} = \frac{3.1\ mL}{dose}$$

3.2 Dosing by Body Surface Area (Humans)

Some medications, including chemotherapeutic agents, require dosing by body surface area (BSA). The dose is expressed as amount of drug per meter squared (m^2). BSA may be calculated using the following formulas:

$$BSA\ (m^2) = \sqrt{[\text{Height (in)} \times \text{Weight (lb)}]/3131}$$

$$BSA\ (m^2) = \sqrt{[\text{Height (cm)} \times \text{Weight (kg)}]/3600}$$

3.2.1 CALCULATING BY BODY SURFACE AREA (HUMAN)

Example—Dosing by BSA (humans)

A physician orders rituximab at a dose of 375 mg/m² every week for 6 weeks for a patient who is 6 ft 2 in tall and weighs 183 lb. Calculate the volume, in mL, of 10-mg/mL rituximab injection needed to make each IV infusion dose as follows:

(a) Calculate the patient's BSA:

$$m^2 = \sqrt{[74\ in \times 183\ lb]/3131} = 2.08\ m^2$$

(b) Multiply the BSA by the dosing rate:

$$2.08\ m^2 \times 375\ mg/m^2 = 780\ mg/dose$$

(c) Calculate the volume of each dose using ratio and proportion:

$$(780\ mg/dose)/(10\ mg/mL) = 78\ mL/dose$$

The preceding calculation may also be completed using DUA as follows:

$$2.08\text{-}m^2\ patient \times \frac{375\ mg}{m^2 \cdot dose} \times \frac{mL}{10\ mg} = \frac{78\ mL}{dose}$$

3.3 Dosing By Body Surface Area (Animals)

BSA for cats and dogs may be calculated using the following formulas. For other animals, consult an appropriate veterinary medicine reference.

Body surface area for cats:

$$BSA\ (m^2) = \{10 \times [body\ weight\ (g)]^{0.667}\}/10{,}000$$

Body surface area for dogs:

$$BSA\ (m^2) = \{10.1 \times [body\ weight\ (g)]^{0.667}\}/10{,}000$$

3.3.1 CALCULATING DOSING BY BODY SURFACE AREA (ANIMALS)

Example—Dosing by BSA (animals)

A veterinarian orders oral cyclophosphamide therapy at a dose of 50 mg/m² for a cat who weighs 5.8 kg. Calculate the dose of cyclophosphamide as follows:

(a) Calculate the cat's BSA:

$$BSA\ (m^2) = [10 \times (5800\ g)^{0.667}]/10{,}000 = 0.324\ m^2$$

(b) Multiply the BSA by the dosing rate:

$$0.324\ m^2 \times 50\ mg/m^2 = 16.2\ mg$$

4. USE OF POTENCY UNITS

Because some substances cannot be completely characterized by chemical and physical means, it may be necessary to express quantities of activity in biological units of potency [see the *USP General Notices and Requirements 5.50.10, Units of Potency (Biological)*].

4.1 Calculating by Use of Potency Units

Examples—Use of potency units

1. Potency units-to-milligrams conversion

A dose of penicillin G benzathine for streptococcal infection is 1.2 million units administered intramuscularly. If a specific product contains 1180 units/mg, calculate the amount, in mg, of penicillin G benzathine in the dose as follows:

$$(1{,}200{,}000\ units)/(1180\ units/mg) = 1017\ mg\ of\ penicillin\ G\ benazathine$$

2. Potency units-to-milligrams conversion

A prescription calls for 60 g of an ointment containing 150,000 units of nystatin per gram. Calculate the quantity of nystatin with a potency of 4400 units/mg that should be weighed for the prescription as follows:

$$60\ g \times (150{,}000\ units\ of\ nystatin/g) = 9{,}000{,}000\ units$$

$$9{,}000{,}000\ units/(4400\ units/mg) = 2045\ mg\ of\ nystatin$$

5. VOLUME AND WEIGHT SUMS

Weights are additive in most mixtures of liquids, semisolids, and solids. Volumes in mixtures of miscible solutions and pure liquids may or may not be additive, based primarily on the effects of volume proportions and intermolecular hydrogen bonding. For example, mixtures containing equal or near-equal volumes of water and ethanol (and other miscible mono-hydroxy alcohols) will be exothermic and result in a volume contraction of <5%, e.g., 50 mL water + 50 mL ethanol yield 97–98 mL at 20°–25°. Negligible volume contraction occurs between water and polyhydroxy or polyhydric alcohols, e.g., glycerin and propylene glycol. Volumes are additive with usually negligible error in aqueous mixtures that contain <10% of mono-hydroxy alcohols, i.e., there is <0.5% volume contraction.

6. DENSITY AND SPECIFIC GRAVITY

Density is defined as the mass of a substance in air at a specific temperature (typically 25°) per unit volume of that substance at the same temperature. Density may be calculated with the following equation:

Density = (mass of substance/volume of substance) at a particular temperature and pressure

Specific gravity (SG) is the unitless ratio of the density of a substance to the density of water at 4°, or [(g of substance/mL)/1.00 g/mL]. Alternatively, SG can be calculated at a particular temperature in some common units of density from density of substance per density of water.

SG may be calculated with the following equation:

SG = (weight of the substance)/(weight of an equal volume of water)

6.1 Calculating Density and Specific Gravity

Examples—Density and specific gravity

1. Density calculation

2.3 g of activated charcoal powder occupies a bulk volume of 5.2 mL at 20° and 1 atm. The density of activated charcoal powder can be calculated as follows:

Density = 2.3 g/5.2 mL = 0.44 g/mL

2. SG calculation

125 g of glycerin occupies a volume of 99 mL at 25°. [NOTE—The density of water at 25° is 0.997 g/mL.] The SG of glycerin can be calculated as follows:

SG = (125 g/99 mL)/(0.997 g/mL) = 1.266

3. Concentrated acid calculation

Hydrochloric acid is approximately a 37% w/w solution of hydrochloric acid in water. Calculate the amount, in g, of hydrochloric acid contained in 75 mL of hydrochloric acid as follows. [NOTE—The SG of hydrochloric acid is 1.18.]

37% w/w × 1.18 = 43.7% w/v

(43.7 g/100 mL) × 75 mL = 32.8 g of hydrochloric acid

7. MILLIEQUIVALENTS AND MILLIMOLES

[NOTE—This section addresses milliequivalents (mEq) and millimoles (mmol) as they apply to electrolytes for dosage calculations. See also the *8. Concentrations Expressions* section of this chapter.]

The quantities of electrolytes administered to patients are usually expressed in terms of mEq. Weight units such as mg or g are not often used for electrolytes because the electrical properties of ions are best expressed as mEq. An equivalent (Eq) is the weight of a substance that supplies 1 unit of charge. An equivalent weight is the weight, in g, of an atom or radical, divided by the valence of the atom or radical. A mEq is 1/1000th of an Eq. The equivalent weight of a compound may be determined by dividing its formula or MW in g by the valence of its largest valence ion.

A mole equals one gram-atomic weight or gram-molecular weight of a substance. A millimole equals 1/1000th of a mole.

7.1 Calculating Milliequivalents and Millimoles

Examples—Milliequivalents and millimoles

1. Calculate the mEq weight of calcium. [NOTE—Calcium has a MW of 40.08, and the valence of calcium is 2^+.]

Eq weight = 40.08 g/2 = 20.04 g

$$\text{mEq weight} = 20.04\ \text{g}/1000 = 0.02004\ \text{g} = 20.04\ \text{mg}$$

2. Calculate the quantity, in mEq, of potassium in a 250-mg Penicillin V Potassium Tablet. [NOTE—Penicillin V potassium has a MW of 388.48 g, there is one potassium atom in the molecule, and the valence of potassium is 1+.]

$$\text{Eq weight} = 388.48\ \text{g}/1 = 388.48\ \text{g}$$

$$\text{mEq weight} = 388.48\ \text{g}/1000 = 0.38848\ \text{g} = 388.48\ \text{mg}$$

$$(250\ \text{mg/tablet})/(388.48\ \text{mg/mEq}) = 0.644\ \text{mEq of potassium/tablet}$$

3. Calculate the mEq of magnesium and sulfate in a 2-mL dose of 50% Magnesium Sulfate Injection. [NOTE—Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) has a MW of 246.47, and the highest valence ion is magnesium 2^+ and sulfate 2^-.]

$$(50\ \text{g}/100\ \text{mL}) \times (2\ \text{mL/dose}) = 1\ \text{g/dose}$$

$$\text{Eq weight} = 246.47\ \text{g}/2 = 123.24\ \text{g/Eq}$$

$$(1\text{g/dose})/(123.24\ \text{g/Eq}) = 0.008114\ \text{Eq} = 8.114\ \text{mEq of both magnesium and sulfate per dose}$$

This problem may also be worked using DUA as follows:

$$\frac{50\ \text{g}}{100\ \text{mL}} \times \frac{2\ \text{mL}}{\text{dose}} \times \frac{2\ \text{Eq}}{246.47\ \text{g}} \times \frac{1000\ \text{mEq}}{\text{Eq}} = \frac{8.114\ \text{mEq}}{\text{dose}}$$

4. A vial of sodium chloride injection contains 3 mEq/mL of sodium chloride. Calculate the strength, in % w/v, of the injection. [NOTE—Sodium chloride has a MW of 58.44.]

$$\frac{3\ \text{mEq}}{\text{mL}} \times \frac{58.44\ \text{g}}{1\ \text{Eq}} \times \frac{\text{Eq}}{1000\ \text{mEq}} = \frac{0.1753\ \text{g}}{\text{mL}}$$

$$(0.1753\ \text{g/mL}) \times 100\ \text{mL} = 17.53\ \text{g in}\ 100\ \text{mL} = 17.53\%\ \text{w/v}$$

5. Calculate the weight of potassium in mmol. [NOTE—Potassium has a MW of 39.1.]
The weight of 1 mol is 39.1 g and the weight in mmol is:

$$39.1\ \text{g}/1000 = 0.0391\ \text{g or}\ 39.1\ \text{mg}$$

6. Calculate the mmol of penicillin V potassium in a 250-mg Penicillin V Potassium Tablet. [NOTE—Penicillin V potassium has a MW of 388.48.]
The weight of 1 mol is 388.48 g, and the weight of 1 mmol is:

$$388.48\ \text{g}/1000 = 0.38848\ \text{g or}\ 388.48\ \text{mg}$$

$$\frac{250\ \text{mg}}{\text{tablet}} \times \frac{\text{mmol}}{388.48\ \text{mg}} = \frac{0.644\ \text{mmol of Penicillin V potassium}}{\text{tablet}}$$

8. CONCENTRATION EXPRESSIONS

The concentration expressions in this section refer to homogeneous mixtures of the following states of matter at a temperature of 20°–30° and pressure of 1 atm (29.92 in Hg, 760 mm Hg, 101.3 kPa, 1013.3 mb): gas in gas, gas in liquid, liquid in liquid, liquid in semisolid, solid in liquid, solid in semisolid, and solid in solid. Concentration expressions used in pharmacy practice and pharmaceutical research include, but are not limited to, those listed in *Table 1*. Common metric drug strength and clinical concentrations include, for example, µg/mL, mg/dL, g or mg per L, and ng/µL (see *General Notices and Requirements 8.240, Weights and Measures*).

Table 1

Title	Abbreviation	Definition
Mass in volume ratios	None is standard	Mass of a dispersed or dissolved ingredient per volume amount of mixtures containing that ingredient
mEq[a] per volume	mEq/volume unit	mEq of an electrolyte or salt per unit of volume of solutions containing that electrolyte or salt

[a] 1 mEq = Eq/1000.
[b] The abbreviation for mole is mol.
[c] 1 mol of solute per 1 kg of solvent is a 1 molal (1 m) solution.
[d] 1 mol of solute per 1 L of solution of that solute is a 1 molar (1 M) solution.
[e] Normality = (Molarity × largest valence ion of a compound), e.g., (18 M H_2SO_4 × 2) = 36 N H_2SO_4, where 2 derives from the 2^- valence of SO_4.
[f] Eq of a compound = (1 mol × largest valence ion of a compound), e.g., 1 mol of lithium citrate = 3 Eq of lithium citrate; 1 mol of $Ca(gluconate)_2$ = 2 Eq of $Ca(gluconate)_2$; and 1 mol of KCl = 1 Eq of KCl.
[g] 1 Eq of solute per 1 L of solution of that solute is a 1 normal (1 N) solution.
[h] *R*, *X*, and *Y* are whole numbers.

Table 1 *(Continued)*

Title	Abbreviation	Definition
Molality	m	mol[b] of a solute/kg of a solvent containing that solute[c]
Molarity	M	mol of a solute/L of a solvent containing that solute[d]
Normality[e]	N	Equivalents (Eq[f]) of a solute/L of a solvent containing that solute[g]
Parts per million	ppm	Parts of a gas, liquid, or solid per 1 million part of another gas, liquid, or solid containing the first gas, liquid, or solid
% Volume in volume	% v/v	mL of liquid per 100 mL of a solvent containing that liquid
% Weight in volume	% w/v	g of a solute per 100 mL of a solvent containing that solute
% Weight in weight	% w/w	g of a solute per 100 g of a mixture containing that solute
Ratio strength	1:*R*	1 part of an ingredient per *R*[h] parts of a mixture containing that ingredient
	1 in *R*	1 part of an ingredient in *R*[h] parts of a mixture containing that ingredient
	X:*Y*	*X*[h] parts of one ingredient per *Y*[h] parts of another ingredient in a mixture

[a] 1 mEq = Eq/1000.
[b] The abbreviation for mole is mol.
[c] 1 mol of solute per 1 kg of solvent is a 1 molal (1 m) solution.
[d] 1 mol of solute per 1 L of solution of that solute is a 1 molar (1 M) solution.
[e] Normality = (Molarity × largest valence ion of a compound), e.g., (18 M H_2SO_4 × 2) = 36 N H_2SO_4, where 2 derives from the 2^- valence of SO_4.
[f] Eq of a compound = (1 mol × largest valence ion of a compound), e.g., 1 mol of lithium citrate = 3 Eq of lithium citrate; 1 mol of Ca(gluconate)$_2$ = 2 Eq of Ca(gluconate)$_2$; and 1 mol of KCl = 1 Eq of KCl.
[g] 1 Eq of solute per 1 L of solution of that solute is a 1 normal (1 N) solution.
[h] *R*, *X*, and *Y* are whole numbers.

8.1 Calculating Normality

Example—Normality

Calculate the amount of sodium bicarbonate powder needed to prepare 50 mL of a 0.07 N solution of sodium bicarbonate ($NaHCO_3$). [NOTE—Sodium bicarbonate has a MW of 84.01.]

In an acid or base reaction, because $NaHCO_3$ may act as an acid by giving up one proton, or as a base by accepting one proton, one Eq of NaHCO3 is contained in each mole of $NaHCO_3$.

$$0.050\ \text{L} \times \frac{0.07\ \text{Eq}}{\text{L}} \times \frac{1\ \text{mol}}{1\ \text{Eq}} \times \frac{84.01\ \text{g}}{1\ \text{mol}} = 0.294\ \text{g of sodium bicarbonate}$$

8.2 Calculating Percentage Concentrations

Percentage concentrations of solutions and other homogeneous mixtures are usually expressed in one of three common forms in which numerator and denominator quantities are in g and mL measurement units.

1. Volume percent (% v/v) = (volume of liquid solute/volume of solution or suspension) × 100
   or % v/v = mL of liquid solute in 100 mL of solution or suspension
2. Weight percent (% w/w) = (weight of solute/weight of mixture) × 100
   or % w/w = g of ingredient in 100 g of mixture
3. Weight in volume percent (% w/v) = (weight of solute/volume of solution or suspension) × 100
   or % w/v = g of solute in 100 mL of solution or suspension

The preceding three equations may be used to calculate any one of the three values (i.e., weights, volumes, or percentages) in a given equation if the other two values are known (see also *General Notices and Requirements 8.140, Percentage Concentrations*).

Examples—Percentage concentrations

1. Weight percent

A prescription order reads as follows (see *Table 2*):

Table 2

Zinc oxide	7.5 g
Calamine	7.5 g
Starch	15 g
White petrolatum	30 g

General Chapters

Calculate the percentage concentration for each of the four components using the preceding weight percent equation as follows:

(a) The total weight of ointment = 7.5 g + 7.5 g + 15 g + 30 g = 60.0 g
(b) The weight percent of zinc oxide = (7.5 g of zinc oxide/60 g of ointment) × 100% = 12.5%
(c) The weight percent of calamine = (7.5 g of calamine/60 g of ointment) × 100% = 12.5%
(d) The weight percent of starch = (15 g of starch/60 g of ointment) × 100% = 25%
(e) The weight percent of white petrolatum = (30 g of white petrolatum/60 g of ointment) × 100% = 50%

2. Volume percent
A prescription order reads as follows:
Rx: Eucalyptus Oil 3% v/v in Mineral Oil.
Dispense 30 mL.
Calculate the quantities of ingredients in this prescription using the volume percent equation as follows:
(a) The amount of eucalyptus oil.

$$3\%\ v/v = (\text{volume of oil in mL}/30.0\ \text{mL}) \times 100\%$$

$$\text{volume in oil} = 0.9\ \text{mL of eucalyptus oil}$$

(b) The amount of mineral oil.

$$30\ \text{mL} - 0.9\ \text{mL} = 29.1\ \text{mL of mineral oil}$$

8.3 Conversions of Concentration Expressions

8.3.1 SOLID-IN-LIQUID SOLUTION CONVERSIONS

The calculations used to convert from percent weight in volume, % w/v, to other concentrations and vice versa, using the same densities and formula or molecular weights, are illustrated as follows for calcium chloride, magnesium sulfate, and potassium chloride solutions in water.

8.3.1.1 Calculating solid-in-liquid conversions

Examples—Solid-in-liquid conversions

1. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to molality (m). [NOTE—Calcium chloride has a MW of 147.01 g; 10% w/v solution has a density of 1.087 g/mL.]

$$10\%\ w/v = 10\ \text{g of calcium}/100\ \text{mL of solution}$$

Using the density of the solution:

$$100\ \text{mL of solution} \times 1.087\ \text{g/mL} = 108.7\ \text{g of solution}$$

$$108.7\ \text{g of solution} - 10\ \text{g of calcium chloride} = 98.7\ \text{g of water} = 0.0987\ \text{kg of water}$$

$$10\ \text{g of calcium chloride}/(147.01\ \text{g of calcium chloride/mol of calcium chloride}) = 0.068\ \text{mol of calcium chloride}$$

$$0.068\ \text{mol of calcium chloride}/0.0987\ \text{kg of water} = 0.689\ \text{m}$$

2. Convert 50% w/v magnesium sulfate ($MgSO_4 \cdot 7H_2O$) to molarity (M). [NOTE—Magnesium sulfate has a MW of 246.47 g.]

$$\frac{50\ \text{g}}{100\ \text{mL}} \times \frac{\text{mol}}{246.47\ \text{g}} \times \frac{1000\ \text{mL}}{1\ \text{L}} = 2.029\ \text{M}$$

3. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to normality (N).

$$\frac{10\ \text{g}}{100\ \text{mL}} \times \frac{1\ \text{mol}}{147.01\ \text{g}} \times \frac{2\ \text{Eq}}{1\ \text{mol}} \times \frac{1000\ \text{mL}}{1\ \text{L}} = 1.36\ \text{N}$$

*2 Eq/mol derived from the 2^+ valence of calcium

4. Convert 10% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to mEq/mL.

$$\frac{10\ \text{g}}{100\ \text{ml}} \times \frac{1\ \text{mol}}{147.01\ \text{g}} \times \frac{2\ \text{Eq}}{1\ \text{mol}} \times \frac{1000\ \text{mEq}}{1\ \text{Eq}} = 1.36\ \text{mEq/mL}$$

5. Convert 0.1% w/v calcium chloride ($CaCl_2 \cdot 2H_2O$) to ppm.

$$(0.1\ \text{g}/100\ \text{mL}) \times (1 \times 10^6\ \text{ppm}) = 1000\ \text{ppm}$$

6. Convert 33% w/v potassium chloride (KCl) to 1:*R* ratio strength.

$$(1/R) = (33 \text{ g}/100 \text{ mL})$$

$$R = 3.03$$

$$1{:}R = 1{:}3$$

8.3.2 LIQUID-IN-LIQUID SOLUTION CONVERSIONS

The calculations used to convert from percent weight in weight, % w/w, and volume in volume, % v/v, to other concentrations and vice versa using the same densities and formula or MWs, are illustrated for glycerin and isopropyl alcohol in water. Besides liquid-in-semisolid, solid-in-semisolid, and solid-in-solid mixtures, % w/w is used for viscous liquids, such as coal tar, glycerin, and concentrated acids.

8.3.2.1 Converting liquid-in-liquid solutions

Examples—Liquid-in-liquid conversions

1. Convert 50% w/w glycerin to % w/v. [NOTE—50% w/w glycerin has a density of 1.13 g/mL.]

$$(50 \text{ g}/100 \text{ g}) \times (1.13 \text{ g/mL}) = 0.565 \text{ g/mL}$$

$$56.5 \text{ g}/100 \text{ mL} = 56.5\% \text{ w/v}$$

2. Convert 70% v/v isopropyl alcohol to % w/w. [NOTE—Isopropyl alcohol has a density of 0.79 g/mL, and 70% v/v isopropyl alcohol has a density of 0.85 g/mL.]

$$70 \text{ mL of isopropyl alcohol} \times (0.79 \text{ g/mL}) = 55.3 \text{ g of isopropyl alcohol}$$

$$100 \text{ mL of solution} \times (0.85 \text{ g/mL}) = 85 \text{ g of solution}$$

$$(55.3 \text{ g of isopropyl alcohol}/85 \text{ g of solution}) \times 100 = 65.06\% \text{ w/w}$$

3. Convert 70% v/v isopropyl alcohol to % w/v. The following values are from example 2.

$$55.3 \text{ g of isopropyl alcohol}/100 \text{ mL of solution} = 55.3\% \text{ w/v}$$

4. Convert 50% w/w glycerin to molality (m). [NOTE—Glycerin has a MW of 92.1.]

$$50 \text{ g of glycerin}/(92.1 \text{ g/mol}) = 0.543 \text{ mol of glycerin}$$

$$100 \text{ g of solution} - 50 \text{ g of glycerin} = 50 \text{ g of water} = 0.05 \text{ kg of water}$$

$$(0.543 \text{ mol of glycerin}/0.05 \text{ kg of water}) = 10.86 \text{ m}$$

5. Convert 70% v/v isopropyl alcohol to molality (m). [NOTE—Isopropyl alcohol has a density of 0.79 g/mL and a MW of 60.1; 70% v/v isopropyl alcohol has a density of 0.85 g/mL.]

$$70 \text{ mL of isopropyl alcohol} \times (0.79 \text{ g/mL}) = 55.3 \text{ g of isopropyl alcohol}$$

$$100 \text{ mL of solution} \times (0.85 \text{ g/mL}) = 85 \text{ g of solution}$$

$$(85 \text{ g of solution} - 55.3 \text{ g of isopropyl alcohol}) = 29.7 \text{ g of water} = 0.0297 \text{ kg of water}$$

$$55.3 \text{ g of isopropyl alcohol}/(60.1 \text{ g/mol}) = 0.92 \text{ mol of isopropyl alcohol}$$

$$(0.92 \text{ mol of isopropyl alcohol}/0.0297 \text{ kg of water}) = 30.98 \text{ m}$$

6. Convert 50% w/w glycerin to molarity (M). [NOTE—Glycerin has a MW of 92.1 g.]

$$\text{From example 1, 50\% w/w glycerin} = 56.5\% \text{ w/v glycerin}$$

$$(56.5 \text{ g}/100 \text{ mL}) \times (\text{mol}/92.1 \text{ g}) \times (1000 \text{ mL/L}) = 6.13 \text{ M}$$

7. Convert 50% w/w glycerin to % v/v. [NOTE—50% w/w of glycerin has a density of 1.13 g/mL; 100% glycerin has a density of 1.26 g/mL.]

$$50 \text{ g of glycerin}/(1.26 \text{ g/mL}) = 39.7 \text{ mL of glycerin}$$

$$100 \text{ g of solution}/(1.13 \text{ g/mL}) = 88.5 \text{ mL of solution}$$

$$(39.7 \text{ mL of glycerin}/88.5 \text{ mL of solution}) \times 100\% = 44.8\% \text{ v/v}$$

9. Convert 50% w/w glycerin to 1 in *R* ratio strength.

$$1/R = (50 \text{ g of glycerin}/100 \text{ g of solution})$$

$$R = 2$$

General Chapters

$$1 \text{ in } R = 1 \text{ in } 2$$

8.3.3 SOLID AND SEMISOLID IN SOLID AND SEMISOLID MIXTURE CONVERSIONS

The calculations used to convert from percent weight in weight (% w/w) to ppm and ratio strengths are illustrated as follows for fluocinonide and tolnaftate in topical semisolids and powders.

8.3.3.1 Calculating solid and semisolid in solid and semisolid mixture conversions

Examples—Solid and semisolid in solid and semisolid mixture conversions

1. Convert 0.05% w/w fluocinonide ointment to ppm.

$$(0.05 \text{ g}/100 \text{ g}) \times (1 \times 10^6 \text{ ppm}) = 500 \text{ ppm}$$

2. Convert 1.5% w/w tolnaftate powder to 1:R ratio strength.

$$1/R = (1.5 \text{ g of tolnaftate}/100 \text{ g of powder})$$

$$R = 67$$

$$1{:}R = 1{:}67$$

3. Convert 1% w/w tolnaftate in talcum powder to $X{:}Y$ ratio strength.

$$100 \text{ g of powder} - 1 \text{ g of tolnaftate} = 99 \text{ g of talcum}$$

$$X{:}Y = 1 \text{ g of tolnaftate}{:}99 \text{ g of talcum}$$

8.4 Dilution and Concentration

A more concentrated solution can be diluted to a lower concentration to obtain appropriate strength and precision when compounding preparations. Powders and semisolid mixtures can be triturated or mixed to achieve lower concentrations. The amount of an ingredient in the diluted mixture is the same as that in the portion of the more concentrated source used to make the dilution; thus, the following equation can be applied to dilution problems $(Q_1)(C_1) = (Q_2)(C_2)$, where Q_1 and Q_2 are the quantity of solutions 1 and 2, respectively, and C_1 and C_2 are concentrations of solutions 1 and 2, respectively. Any quantities and concentration terms may be used but the units of those terms must be the same on both sides of the equation.

8.4.1 CALCULATING DILUTION AND CONCENTRATION

Examples—Dilutions and fortifications

1. Semisolid dilution

Calculate the quantity (Q_2), in g, of diluent that must be added to 60 g of a 10% w/w ointment to make a 5% w/w ointment.

$$(Q_1) = 60 \text{ g}, (C_1) = 10\% \text{ w/w, and } (C_2) = 5\% \text{ w/w}$$

$$60 \text{ g} \times 10\% \text{ w/w} = (Q_2) \times 5\% \text{ w/w}$$

$$(Q_2) = 120 \text{ g}$$

$$120 \text{ g} - 60 \text{ g} = 60 \text{ g of diluent to be added}$$

2. Solid dilution

Calculate the amount of diluent that should be added to 10 g of a trituration (1 in 100) to make a mixture that contains 1 mg of drug in each 10 g of the final mixture.

$$\text{Convert mg to g: } 1 \text{ mg of drug} = 0.001 \text{ g of drug}$$

$$10 \text{ g of mixture should contain } 0.001 \text{ g of drug}$$

$$(Q_1) = 10 \text{ g}, (C_1) = (1 \text{ in } 100), \text{ and } (C_2) = (0.001 \text{ in } 10)$$

$$10 \text{ g} \times (1/100) = (Q_2) \times (0.001/10)$$

$$(Q_2) = 1000 \text{ g}$$

Because the final mixture of 1000 g contains 10 g of the trituration, 990 g (or 1000 g − 10 g) of diluent is required to prepare the mixture at a concentration of 0.001 g of drug in each 10 g.

3. Liquid dilution

Calculate the percentage strength (C_2) of a solution obtained by diluting 400 mL of a 5.0% w/v solution to 800 mL.

General Chapters

$$(Q_1) = 400 \text{ mL}, (C_1) = 5.0\% \text{ w/v, and } (Q_2) = 800 \text{ mL}$$

$$400 \text{ mL} \times 5\% \text{ w/v} = 800 \text{ mL} \times (C_2)$$

$$(C_2) = 2.5\% \text{ w/v}$$

4. Liquid fortification

Calculate the additional amount, in g, of codeine phosphate that need to be added to 180 mL of a 12 mg/5 mL elixir of acetaminophen with codeine to have a final concentration of 30 mg/5 mL of codeine phosphate.

Amount to add = Total amount required − Amount present

Total amount required: (30 mg/5 mL) × 180 mL = 1080 mg of codeine phosphate

Amount present = (12 mg of codeine/5 mL) × 180 mL = 432 mg of codeine phosphate

Amount to add: 1080 mg − 432 mg = 648 mg of codeine phosphate

9. ALCOHOL

To achieve compliance with the statements in the *General Notices and Requirements* about Alcohol and the *USP* monograph for *Alcohol*, some conventions and special calculations are needed. See *General Notices and Requirements 5.20.20.1 In Compounded Preparations*, *8.30 Alcohol Content*, and *Labeling* ⟨7⟩, *Alcohol* for information. The *USP* monograph for *Alcohol* states that it contains 92.3%–93.8% by weight corresponding to 94.9%–96.0% by volume of alcohol (C_2H_5OH) at 15.56°. The percent concentration for alcohol is generally taken to be 95% v/v of alcohol (C_2H_5OH) in water.

In summary:

- When the word alcohol is written on a prescription order or in a formula, as for example "alcohol 10 mL" or "dissolve in 5 mL of alcohol", the compounder should use the Alcohol, USP [that is 95% alcohol (C_2H_5OH)].
- When the word alcohol is written with a percent, for example "alcohol 20%", this means 20% v/v of alcohol (C_2H_5OH). If this percent is on a label of a commercial product, it means the product contains 20% v/v alcohol (C_2H_5OH). If this is part of a compounding formula, it means the compounder must add the equivalent of 20% v/v alcohol (C_2H_5OH), which may require special calculations.
- Labels of products and compounded preparations are to include the content of alcohol (C_2H_5OH) in % v/v. For compounded preparations, this value must often be calculated based on the volume(s) of alcohol-containing ingredients added.

For calculations when preparing compounded drug preparations using Alcohol, USP, the first step is to determine the quantity, in mL, of alcohol needed, and the second step is to determine the % v/v of alcohol (C_2H_5OH) in the final preparation so that it can be properly labeled.

9.1 Calculating Alcohol

Examples—Alcohol

1. Determine the quantity of alcohol needed for the prescription (see *Table 3*):

Table 3

Clindamycin	1%
Alcohol	15%
Propylene glycol	5%
Purified water, a sufficient quantity to make	60 mL

(a) In this prescription order, the alcohol 15% means the preparation contains 15% v/v of alcohol (C_2H_5OH).

(b) For 60 mL of preparation, calculate the quantity of alcohol (C_2H_5OH) needed:

$$15\% \text{ v/v} \times 60 \text{ mL} = 9 \text{ mL of alcohol}$$

(c) Because the source of alcohol (C_2H_5OH) is Alcohol, USP, calculate the volume, in mL, of Alcohol, USP needed to give 9 mL of alcohol (C_2H_5OH):

$$9 \text{ mL alcohol}/95\% \text{ v/v Alcohol, USP} = 9.5 \text{ mL of Alcohol, USP}$$

Therefore, add 9.5 mL of Alcohol, USP to this preparation.

(d) Determine the % v/v alcohol content for labeling.

Because labeling of alcohol is in % v/v of alcohol (C_2H_5OH), the alcohol content of this preparation would be labeled: Alcohol 15%.

2. Determine the alcohol content, in % v/v, for the prescription (see *Table 4*):

Table 4

Castor oil	40 mL
Acacia	As needed
Alcohol	15 mL
Cherry syrup	20 mL
Purified water, a sufficient quantity to make	100 mL

Because the *USP* monograph for *Alcohol* is to be used when alcohol is called for in formulas, measure 15 mL of Alcohol, USP.

$$95\% \text{ Alcohol, USP} \times 15 \text{ mL} = 14.25 \text{ mL of alcohol}$$

$$14.25 \text{ mL of alcohol}/100 \text{ mL of preparation} = 14.25\% \text{ alcohol}$$

10. ALLIGATION ALTERNATE AND ALGEBRA METHODS

10.1 Alligation Alternate

Alligation is a method of determining the proportions in which substances of different strengths are mixed to yield a desired strength or concentration. Once the proportion is found, the calculation may be performed to find the exact amounts of substances required.

Set up the problem as follows.

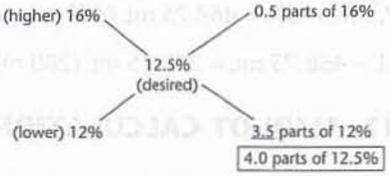
1. Place the desired percentage or concentration in the center.
2. Place the percentage of the substance with the lower strength on the lower left-hand side.
3. Place the percentage of the substance with the higher strength on the upper left-hand side.
4. Subtract the lower percentage from the desired percentage, and place the obtained difference on the upper right-hand side.
5. Subtract the desired percentage from the higher percentage, and place the obtained difference on the lower right-hand side.

The results on the right side determine how many parts of the two different percentage strengths should be mixed to produce the desired percentage strength of a drug mixture. The total parts will equal the final weight or volume of the preparation.

10.1.1 CALCULATING BY USING THE ALLIGATION ALTERNATE

Examples—Alligation alternate

1. Determine the amount of ointment containing 12% drug concentration and the amount of ointment containing 16% drug concentration must be used to make 1 kg of a preparation containing a 12.5% drug concentration.



In a total of 4 parts of 12.5% preparation, 3.5 parts of 12% ointment and 0.5 parts of 16% ointment are needed.

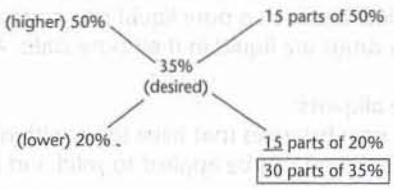
4 parts correspond to 1 kg or 1000 g.

1 part corresponds to 250 g.

3.5 parts correspond to 3.5 × 250 g or 875 g of 12% ointment.

0.5 parts correspond to 0.5 × 250 g or 125 g of 16% ointment.

2. Determine the volume, in mL, of 20% dextrose in water and 50% dextrose in water needed to make 750 mL of 35% dextrose in water.



In a total of 30 parts of 35% dextrose in water, 15 parts of 50% dextrose in water and 15 parts of 20% dextrose in water are required.

30 parts correspond to 750 mL.

15 parts correspond to 375 mL.

Thus, use 375 mL of the 20% solution and 375 mL of the 50% solution to prepare the preparation.

10.2 Algebra Method

The following algebraic equation may be used instead of alligation to solve problems of mixing two different strengths of the same ingredient:

$(C_s \times Q_s) + (C_w \times Q_w) = (C_f \times Q_f)$, where *C* is concentration or strength, *Q* is the quantity; and the subscript *s* identifies the strongest strength, *w* identifies the weakest strength, *f* represents the final mixture with a strength less than *s* and greater than *w*, $(Q_s + Q_w) = Q_f$, $Q_s = (Q_f - Q_w)$, and $Q_w = (Q_f - Q_s)$.

10.2.1 CALCULATING BY USING THE ALGEBRA METHOD

Examples—Algebra method

1. Determine the amount, in g, of 16% w/w drug ointment and 12% w/w drug ointment required to prepare 1 kg of 12.5% w/w drug ointment.

$$(16\% \times Q_s) + [12\% \times (1000\text{ g} - Q_s)] = 12.5\% \times 1000\text{ g}$$

$$16\%\ Q_s + 120\text{ g} - 12\%\ Q_s = 125\text{ g}$$

$$4\%\ Q_s = 5\text{ g}$$

$$Q_s = 5\text{ g}/4\% = 125\text{ g of 16\% ointment}$$

$$Q_w = 1000\text{ g} - 125\text{ g} = 875\text{ g of 12\% ointment}$$

2. Determine the volume, in mL, of 10% dextrose injection and 50% dextrose injection needed to make 750 mL of 35% dextrose injection.

$$(50\% \times C_s) + [10\% \times (750\text{ mL} - C_s)] = 35\% \times 750\text{ mL}$$

$$50\%\ C_s + 75\text{ mL} - 10\%\ C_s = 262.5\text{ mL}$$

$$40\%\ C_s = 187.5\text{ mL}$$

$$C_s = 187.5\text{ mL}/40\% = 468.75\text{ mL (470 mL practically)}$$

$$C_w = 750\text{ mL} - 468.75\text{ mL} = 281.25\text{ mL (280 mL practically)}$$

11. ALIQUOT CALCULATIONS

When the quantity of drug desired requires a degree of precision in measurement that is beyond the capability of the available measuring devices, the pharmacist may use the aliquot method of measurement. It applies when potent drug substances are compounded, or when the total amount of the active drug in a single dose or individualized doses is less than the minimum accurately weighable quantity (MAWQ). Even if the amount of drug needed is greater than the MAWQ per unit, an aliquot will provide more material per unit, which will aid in handling and administration. Aliquot means "containing an exact number of times in something else"; the aliquot must be a proportional part of the total. Therefore, 5 is an aliquot part of 15, because 5 is contained exactly 3 times in 15. Both the total volume of solution or weight of powder triturate and the aliquot volume/weight should be easily and accurately measurable. If the solution or powder triturate is highly concentrated and a small error is made in measuring the aliquot, a large error can occur in the quantity of drug brought to the final formulation.

Aliquots can be: solid–solid, when the active drug and the diluents are solids; solid–liquids, when the active drug is solid and is to be incorporated into a liquid preparation, such as a solution, an emulsion, or a suspension; and liquid–liquid, when the active drug is liquid and the diluents are liquids. It can be a pure liquid or a concentrated solution of a drug. Aliquots of pure liquids are relatively uncommon because few drugs are liquid in their pure state. Aliquots involving concentrated solutions are more common.

There are two general methods to prepare aliquots:

1. Aliquot method 1 is applicable to drugs or substances that have to be within the degree of accuracy provided by the measuring device. It is the simplest method and can be applied to solid and liquid aliquots.

2. Aliquot method 2, also known as the dilution factor method, is useful when there is more flexibility in the amount of drug that may be measured.

Aliquot Method 1:

(a) The MAWQ amount of drug is measured.

(b) The drug is diluted with an arbitrary amount of diluent.

(c) The amount of dilution that will give the desired amount of drug is calculated, and the amount is measured.

Aliquot Method 2:

(a) The quantity of drug to be measured is determined by multiplying the amount of drug needed by an appropriately determined factor, called the dilution factor. The dilution factor must be a whole number more than or equal to the MAWQ divided by the amount of drug needed.

(b) An arbitrary amount of diluent is measured and added. The amount of diluent used can be determined by different methods, provided the amount of diluent chosen will give an aliquot greater than or equal to the MAWQ.

(c) The amount of aliquot needed is determined by multiplying the weight or volume of the dilution by the inverse of the dilution factor. Dilution factors are usually chosen to be whole numbers.

The general calculations can be shown as:

$$A/B = C/D$$

A = amount of drug desired
B = amount of drug measured
C = amount of drug in aliquot
D = aliquot total amount

11.1 Calculating Aliquots

Examples—Aliquots

1. Solid-in-liquid dilution (Aliquot Method 1)

Prepare 100 mL of a solution containing 0.2 mg/mL of clonidine using water as the diluent. To prepare this solution, 20 mg of clonidine is needed.

(a) Select the weight of drug desired (A) to be equal to or greater than the MAWQ. In this situation, the MAWQ of the balance is 120 mg.

(b) Select the aliquot volume (D) in which the desired amount of drug (C) will be contained. This establishes the concentration of the solution to be prepared. Clonidine solubility is 1 g/13 mL, so if 5 mL is selected as the aliquot volume, the concentration in that solution will be 20 mg/5 mL. Therefore, solubility will not be a problem in this aqueous solution.

(c) Using the preceding formula, calculate the volume of solution (B) to be prepared.

$$120 \text{ mg of clonidine}/B = 20 \text{ mg of clonidine}/5 \text{ mL of aliquot}$$

$$B = 30 \text{ mL}$$

(d) Prepare the solution containing 120 mg of clonidine in 30 mL of *Purified Water*. Transfer a 5-mL aliquot from this solution to a final container, and add sufficient *Purified Water* to bring the formulation to a final volume of 100 mL.

2. Solid-in-solid dilution (Aliquot Method 2)

Prepare an individual dose of codeine phosphate 20 mg.

(a) Select a dilution factor that will yield a quantity that is greater than or equal to the MAWQ, and weigh this amount. In this case, the dilution factor may be greater than or equal to 6 because 6 × 20 mg = 120 mg. The smallest dilution factor that may be chosen is 6 if the MAWQ of the balance is 120 mg.

(b) Weigh an amount of diluent that will give an aliquot greater than or equal to the MAWQ. In this example, 600 mg of diluent is weighed.

(c) Mix the two powders thoroughly by geometric trituration in a mortar.

(d) Calculate the total weight of the dilution: 120 mg codeine phosphate + 600 mg diluent = 720 mg.

(e) Calculate the aliquot part of the dilution that contains 20 mg of codeine phosphate by multiplying the total weight of the dilution by the inverse of the dilution factor: 720 mg × (1/6) = 120 mg.

(f) Weigh this calculated amount of the dilution (120 mg) to get the desired 20 mg of codeine phosphate per dose.

12. POWDER VOLUME CALCULATIONS

12.1 Displacement in Suspension

12.1.1 CALCULATING POWDER VOLUME

Examples—Powder volume

1. Powder displacement in suspension

The directions to reconstitute a 150 mL bottle of an amoxicillin for oral suspension of 250 mg/5 mL require 111 mL of *Purified Water*. The physician has requested that the product be reconstituted at a concentration of 500 mg/5 mL. Calculate the amount of *Purified Water* required for the higher concentration.

(a) Calculate the volume of the suspension occupied by the amoxicillin powder:

$$150\ \text{mL} - 111\ \text{mL} = 39\ \text{mL}$$

(b) Calculate the quantity of amoxicillin present in the entire bottle:

$$150\ \text{mL} \times (250\ \text{mg}/5\ \text{mL}) = 7500\ \text{mg}$$

(c) Calculate the total volume of the suspension at the requested concentration (500 mg/5 mL):

$$7500\ \text{mg}/(500\ \text{mg}/5\ \text{mL}) = 75\ \text{mL}$$

(d) Calculate the volume of *Purified Water* needed to reconstitute the powder by subtracting the powder volume calculated in step a:

$$75\ \text{mL} - 39\ \text{mL} = 36\ \text{mL of } \textit{Purified Water}$$

NOTE—Such formulations may be too viscous to flow freely.

2. Powder volume in drugs for injection

If the powder volume of 250 mg of ceftriaxone for injection is 0.1 mL, calculate the amount of diluent that should be added to 500 mg of ceftriaxone for injection to make a suspension with a concentration of 250 mg/mL.

(a) Calculate the total volume of injection:

$$500\ \text{mg}/(250\ \text{mg/mL}) = 2\ \text{mL}$$

(b) Calculate the volume occupied by 500 mg of ceftriaxone for injection:

$$500\ \text{mg}/(250\ \text{mg}/0.1\ \text{mL}) = 0.2\ \text{mL}$$

(c) Calculate the volume of the diluent required:

$$(2\ \text{mL of suspension}) - (0.2\ \text{mL of ceftriaxone for injection}) = 1.8\ \text{mL of diluent}$$

13. INTRAVENOUS FLOW OR INFUSION RATES

Intravenous (IV) solutions and emulsions may be administered by gravity flow and infusion or syringe pumps. Gravity-flow IV sets are regulated by an adjustable clamp on the tubing, and the approximate flow rate is determined by counting the number of drops per 10–15 seconds, then adjusting that to a per minute rate. Manufactured IV sets are typically calibrated to deliver from 15 to 60 drops/mL, depending on the particular set.

13.1 Solving by Multiple or Separate Steps

As in previous sections, the following examples may be solved by multiple separate steps, or a single-DUA procedure.

13.1.1 CALCULATING INTRAVENOUS FLOW OR INFUSION RATES

Examples—IV or infusion rates

1. An IV infusion of dextrose 5% in water with 20 mEq of potassium chloride is to be administered to a 6-year-old child at the rate of 12 mL/hour. An IV administration set that delivers 60 drops/mL is available. Calculate the flow rate in drops/minute:

$$\frac{12\ \text{mL}}{\text{hour}} \times \frac{60\ \text{drops}}{\text{mL}} \times \frac{\text{hour}}{60\ \text{minutes}} = \frac{12\ \text{drops}}{\text{minute}}$$

2. A 63.6-kg patient is admitted to the Emergency Department and requires a dopamine hydrochloride infusion to maintain an adequate blood pressure. The drug is ordered at an initial rate of 2 µg/kg/minute. A 400-mg/250-mL dopamine hydrochloride injection is available. Calculate the flow rate in mL/hour to be administered by infusion pump:

$$63.6\ \text{kg} \times \frac{2\ \mu\text{g}}{\text{kg} \cdot \text{minute}} \times \frac{250\ \text{mL}}{400\ \text{mg}} \times \frac{\text{mg}}{1000\ \mu\text{g}} \times \frac{60\ \text{minutes}}{\text{hour}} = \frac{4.8\ \text{mL}}{\text{hour}}$$

14. ISOOSMOTIC SOLUTIONS

The following discussion and calculations have therapeutic implications in preparations of dosage forms intended for ophthalmic, subcutaneous, intravenous, and intrathecal administration as well as for neonatal use.

14.1 Tonicity

Cells of the body, such as erythrocytes, will neither swell nor shrink when placed in a solution that is isotonic with body fluids. The measurement of tonicity, however, which is a physiological property, is somewhat difficult. A 0.9% w/v sodium chloride injection, which has a freezing point (FP) of −0.52°, is both isotonic and isoosmotic with body fluids. In contrast to isotonicity, FP depression is a physical property. Some solutions that are isoosmotic with body fluids are not isotonic, because they contain solutes to which cells are freely permeable rather than semipermeable. Freely permeable solutes (e.g., boric acid and urea) can cause erythrocyte lysis, i.e., behave as if they were hypotonic in concentrations that are hyperosmotic relative to body fluids. Nevertheless, many pharmaceutical products are prepared using FP data or related sodium chloride data to prepare solutions that are isoosmotic with body fluids. A closely related topic is osmolarity (see chapter *Osmolality and Osmolarity* ⟨785⟩).

FP data or sodium chloride equivalents of pharmaceuticals and excipients (see *Table 5*) may be used to prepare isoosmotic solutions, as shown in the following examples.

14.1.1 CALCULATING TONICITY

Example—Tonicity

Determine the amount of sodium chloride (NaCl) required to prepare 60 mL of an isoosmotic solution of atropine sulfate injection 0.5% using the E values and the FP depression values in *Table 5*.

Table 5. Sodium Chloride Equivalents (E) and FP Depressions for a 1% Solution of the Drug or Excipient

Drug or Excipient	E	FP Depression
Atropine sulfate	0.13	0.075
Sodium chloride	1.00	0.576

Using the E values:

(a) The total amount of substances equivalent to a 0.9% sodium chloride injection = (0.9 g/100 mL) × 60 mL = 0.54 g.
(b) The amount of atropine sulfate required = (0.5 g/100 mL) × 60 mL = 0.3 g.
(c) 1 g of atropine sulfate is equivalent to 0.13 g of sodium chloride.
(d) 0.3 g of atropine sulfate is equivalent to 0.3 × 0.13 g = 0.039 g of sodium chloride.
(e) Thus, the required amount of sodium chloride is 0.54 g − 0.039 g = 0.501 g or 0.5 g.

Using FP depression values:

(a) The FP depression required is 0.52°.
(b) A 1% solution of atropine sulfate causes an FP depression of 0.075°.
(c) A 0.5% solution of atropine sulfate causes an FP depression of 0.5 × 0.075° = 0.0375°.
(d) The additional FP depression required is 0.52° − 0.0375° = 0.483°.
(e) A 1% solution of sodium chloride causes an FP depression of 0.576°.
(f) Therefore, an FP depression of 1° is caused by a 1%/0.576 = 1.736% solution of sodium chloride.
(g) 1.736% × 0.483 = 0.838% solution of sodium chloride causes an FP depression of 0.482°.
(h) The required amount of sodium chloride is (0.838%) × 60 mL = 0.502 g or 0.5 g.

15. pH AND BUFFER CALCULATIONS

15.1 pH Calculations

See *Appendix 1* for logarithmic definitions and applications.

pH = −log $[H_3O^+]$, and pKa = −log ($[H_3O^+][A^-]/[HA]$), where $[H_3O^+]$ is the hydrodium ion concentration in an aqueous solution, $[A^-]$ is the ionic form of the relevant acid, and Ka is the ionization constant of either a monoprotic acid or a particular proton from a polyprotic acid in aqueous solution. The $[H^+]$ = the antilogarithm of (−pH) or 10^{-pH}; and Ka = the antilogarithm of (−pKa) or 10^{-pKa}.

The pH of an aqueous solution containing a weak acid may be calculated using the Henderson–Hasselbalch equation:

$$pH = pKa + \log [(\text{base form})/(\text{acid form})]$$

The buffer equation symbol (↔) represents the equilibrium between conjugate base and acid forms or pairs of the same molecule. It is called the buffer equation, because small changes in the ratio of concentrations of the conjugate forms result in a logarithmically smaller change in pH. The salt form can be an acid or base, depending on structure; thus, its conjugate form is a base or acid, respectively.

Example 1:

B and BH^+ represent a nonionized or "free" base and cationic acid pair, $BH^+ \leftrightarrow B + H^+$

Example 2:

HA and A^- represent a nonionized or "free" acid and anionic base pair, $HA \leftrightarrow A^- + H^+$

General Chapters

Example 3:
H_nA^- and $H_{n-1}A^{2-}$, such as $H_2PO_4^-$ and HPO_4^{2-}, represent an anionic acid and anionic base relative to each other; the pKa = 7.2 for $H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+$.

15.1.1 CALCULATING pH

Example—pH

A solution contains 0.020 mol/L of sodium acetate and 0.010 mol/L of acetic acid, which has a pKa value of 4.76. Calculate the pH and the $[H^+]$ of the solution as follows:

$$pH = 4.76 + \log (0.020/0.010) = 5.06$$

$$[H^+] = \text{antilogarithm of } (-5.06) = 8.69 \times 10^{-6}$$

15.2 Buffer Solutions

15.2.1 DEFINITION

A buffer solution is an aqueous solution that resists a change in pH when small quantities of acid or base are added, when diluted with the solvent, or when the temperature changes. Most buffer solutions are mixtures of a weak acid and one of its salts, or mixtures of a weak base and one of its salts. Water and solutions of a neutral salt, such as sodium chloride, have very little ability to resist the change of pH and are not capable of effective buffer action.

15.2.2 PREPARATION, USE, AND STORAGE OF BUFFER SOLUTIONS

Buffer solutions for Pharmacopeial tests should be prepared using freshly boiled and cooled water (see *Reagents, Indicators and Solutions*). They should be stored in containers such as Type I glass bottles and used within 3 months of preparation.

Buffers used in physiological systems are carefully chosen so as to not interfere with the pharmacological activity of the medicament or the normal function of the organism. Commonly used buffers in parenteral products, for example, include: the nonionized acid and base salt pairs of acetic acid and sodium acetate, citric acid and sodium citrate, glutamic acid and sodium glutamate, and monopotassium or monosodium phosphate and dipotassium or disodium phosphate; and the acid salt and nonionized base pair tris(hydroxymethyl)aminomethane hydrochloride and tris(hydroxymethyl)aminomethane. Buffer solutions should be freshly prepared.

The Henderson–Hasselbalch equation, noted in *15.1 pH Calculations*, allows calculation of the pH and concentrations of conjugate pairs of weak acids and their salts and weak bases and their salts in buffer solutions when the pKa of the acid form of the buffer pair is known. Appropriately modified, this equation may be applied to buffer solutions composed of a weak base and its salt.

15.2.3 BUFFER CAPACITY

The buffer capacity of a solution is the measurement of the ability of that solution to resist a change in pH upon addition of small quantities of a strong acid or base. An aqueous solution has a buffer capacity of 1 when 1 L of the buffer solution requires 1 g equivalent of strong acid or base to change the pH by 1 unit. Therefore, the smaller the pH change upon the addition of a specified amount of acid or base, the greater the buffer capacity of the buffer solution. Usually, in analysis, much smaller volumes of buffer are used to determine the buffer capacity. An approximate formula for calculating the buffer capacity is g equivalents of strong acid or base added per L of buffer solution per unit of pH change, i.e., (g equivalents/L)/(pH change).

15.2.4 CALCULATING BUFFER CAPACITY

Example—Buffer capacity

The addition of 0.01 g equivalents of sodium hydroxide to 0.25 L of a buffer solution produced a pH change of 0.50. The buffer capacity of the buffer solution is calculated as follows:

$$(0.01 \text{ Eq}/0.25 \text{ L})/0.50 \text{ pH change} = 0.08(\text{Eq/L})/(\text{pH change})$$

16. TEMPERATURE

The relationship between Celsius or Centigrade (°C) and Fahrenheit (°F) temperature scale is expressed by the following equations:

$$°C = (°F - 32) \times (5/9)$$

$$°F = (°C \times 1.8) + 32$$

16.1 USP Temperatures

According to the *General Notices and Requirements 8.180 Temperatures*, temperatures are expressed in centigrade (Celsius) degrees, and all other measurements are made at 25° unless otherwise indicated. For instructional purposes, °F is shown in the examples.

16.1.1 CALCULATING TEMPERATURES

Examples—Temperatures

1. Convert 77°F to Celsius degrees.

$$°C = (77°F - 32) \times (5/9) = 25°C$$

2. Convert 30°C to Fahrenheit degrees.

$$°F = (30°C \times 1.8) + 32 = 86°F$$

The relationship between the Kelvin or absolute (K) and the Celsius (°C) scales is expressed by the equation:

$$K = °C + 273.1$$

17. ENDOTOXINS

An endotoxin is a lipopolysaccharide that comes from a particular source, where species and strain number are usually indicated.

17.1 Endotoxin Concentrations

For more information concerning endotoxins, see *Bacterial Endotoxins Test* ⟨85⟩.

17.1.1 CALCULATING ENDOTOXINS

Example—Endotoxins

A 71.8-kg patient is to receive an intrathecal infusion of morphine sulfate at a rate of 0.3 mg/hour. The solution will be prepared by diluting preservative-free morphine sulfate injection, which contains 10 mg/mL of morphine sulfate, with 0.9% sodium chloride injection to produce an infusion rate of 2 mL/h.

1. Determine the volume, in mL, of morphine sulfate injection (10 mg/mL) and 0.9% sodium chloride injection needed to prepare a 24-hour infusion.

0.3 mg of morphine sulfate/hour × 24 hours = 7.2 mg of morphine sulfate

7.2 mg of morphine sulfate/(10 mg/mL) = 0.72 mL of morphine sulfate injection

2 mL of infusion/h × 24 h = 48 mL of total volume

48 mL total volume − 0.72 mL morphine sulfate injection = 47.28 mL of 0.9% sodium chloride injection

2. Calculate the maximum potential endotoxin load/hour for this preparation. [NOTE—*USP* monographs specify upper limits of 14.29 USP Endotoxin Units (EU)/mg of morphine sulfate in injections for intrathecal use, and 0.5 EU/mL for injections containing 0.5%–0.9% sodium chloride.]

7.2 mg of morphine sulfate injection × 14.29 EU/mg of morphine sulfate = 102.89 EU from morphine sulfate

47.28 mL of sodium chloride injection × 0.5 EU/mL = 23.64 EU from 0.9% sodium chloride injection

Endotoxin load = 102.89 EU + 23.64 EU = 126.53 EU

126.53 EU/24 hour = 5.27 EU/hour

3. Determine if the endotoxin load in step 2 exceeds the allowable USP limit for this patient. [NOTE—The maximum endotoxin load by intrathecal administration is 0.2 EU/kg/hour (see chapter ⟨85⟩).]

Maximum endotoxin load = (0.2 EU/kg/hour) × 71.8 kg-patient = 14.36 EU/hour

The endotoxin load of 5.27 EU/hour does not exceed the allowable limit of 14.36 EU/hour.

18. STABILITY AND EXPIRATION DATE CALCULATIONS

18.1 Stability Based on Rate Calculations

Calculation of a predetermined minimum percentage of initial drug strength or other quality parameter, e.g., in vitro dissolution of active pharmaceutical ingredients (APIs) or active drugs in solid oral dosage forms, is based on component-specific assays and other validated scientific testing. The expiration date or time elapsed until such minimum acceptable limits are reached for a specific manufactured product is exclusive to the specific formulation, packaging, and environmental conditions, e.g., temperature, humidity, and illumination, to which the item is subjected. See also chapters ⟨659⟩, ⟨795⟩, ⟨797⟩, ⟨1163⟩, and ⟨1191⟩.

The degradation or concentration loss rates or kinetics of most APIs can be accurately represented or modeled by either zero order (constant) or first order (mono-exponential) rate equations. Zero order calculations are generally applicable to solids, semisolids, suspensions in which a majority of the drug strength is present as solid particles, and auto-oxidation in solutions. First order calculations are generally applicable for drug hydrolysis in solutions.

18.2 Zero Order Rate Calculations

The isothermal zero order or constant rate equation for a particular formulation is $C = C_0 - kt$, where C is the concentration of API at any time, C_0 is the concentration at origination or time zero, k is the reaction rate constant, and t is any time after origination or zero. The values and units of the rate, dC/dt, and rate constant, k, are the same for zero order processes, i.e., the units are concentration/time, such as mg/mL/day.

18.2.1 ZERO ORDER RATE EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate calculations of the zero order rate equation from original concentration assay and time data, and an expiration date using that equation.

18.2.1.1 Calculating zero order rate

Examples—Zero order rate

1. Calculate the zero order rate equation based on the assay results for a drug suspension at 25° (see *Table 6*):

Table 6

C (mg/mL)	*t* (days)
49	3
47.5	8
44.8	17
42.3	26

Linear regression of the *C* (ordinate) versus *t* (abscissa) values yields the equation, $C = 49.84 - 0.292t$ with a correlation coefficient of 0.9996.

2. Calculate the time when $C = 0.9 \times C_0$, i.e., the expiration date where the concentration will be 90% of the original concentration (t_{90}):

$$C = 49.84 - 0.292t$$

$$0.9 \times 49.84 = 49.84 - 0.292(t_{90})$$

$$t_{90} = (44.86 - 49.84)/-0.292 = 17.05 \text{ days}$$

3. Using the previous linear regression equation, calculate the C of the drug suspension at 25° when $t = 12$ days:

$$C = 49.84 - (0.292 \times 12) = 46.34 \text{ mg/mL}$$

4. Calculate t when $C = 45$ mg/mL:

$$45 = 49.84 - 0.292t$$

$$t = (45 - 49.84)/-0.292 = 16.6 \text{ days}$$

18.2.2 ZERO ORDER VALUES CALCULATED FROM A RATE EQUATION

The following are examples of expiration dates calculated from a rate equation derived from original concentration assay and time data.

18.2.2.1 Calculating zero order values from a rate equation

Examples—Zero order from a rate equation

General Chapters

1. Calculate the t_{80} expiration date of a drug cream at 25° using the equation, $C = 0.05 - 0.0003t$, where the C unit is % w/w and the t unit is months. At t_{80}, $C = 0.8C_0$.

$$0.8 \times 0.05 = 0.05 - 0.0003(t_{80})$$

$$t_{80} = 33.3 \text{ months}$$

2. Calculate the t_{80} expiration date of the drug cream formulation in example 1, but for which C_0 is 0.1:

$$0.8 \times 0.1 = 0.1 - 0.0003(t_{80})$$

$$t_{80} = 66.7 \text{ months}$$

18.3 First Order Rate Calculations

The isothermal first order rate equation for a particular formulation in exponential form is $C = C_0e^{-kt}$, and in linear form is $\ln(C) = \ln(C_0) - kt$, where C is the concentration of an API at any time, C_0 is the concentration at origination or time zero, k is the reaction rate constant, and t is any time after origination or zero. The constantly changing rate, dC/dt, and rate constant, k, are not the same for first order processes. The rate units are concentration/time, e.g., mg/mL/hour, but the rate constant unit is reciprocal time, time^{-1}, e.g., hour^{-1}.

18.3.1 FIRST ORDER LINEAR RATE EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate calculation of the linear first order rate equation from original concentration assay and time data and calculation of an expiration date using that equation.

18.3.1.1 Calculating first order linear rate equations

Example—First order linear rate

1. Calculate the linear first order rate equation based on the assay results for a drug solution at 27° (see *Table 7*):

Table 7

C (mg/mL)	t (hour)
12.3	2
11.9	6
11.5	14
10.6	24

Linear regression of the ln(C) (ordinate) versus t (abscissa) values yields the equation, $\ln(C) = 2.522 - 0.0065t$ with a correlation coefficient of 0.992.

2. From the linear regression equation, calculate the time when 95% of the original concentration is reached, t_{95}, when $C = 0.95C_0$, which is the predetermined expiration date:

$$\ln(C_0) = 2.522; \text{ thus, } C_0 = e^{2.522} = 12.45 \text{ mg/mL}$$

$$\ln(0.95 \times 12.45) = 2.522 - 0.0065(t_{95})$$

$$t_{95} = (2.470 - 2.522)/-0.0065 = 8 \text{ hours}$$

18.3.2 FIRST ORDER VALUES CALCULATED FROM A LINEAR EQUATION

The following are examples of an expiration date, concentration, and time calculated for the same drug solution at 22° from the rate equation, $\ln(C) = 4.382 - 0.076t$, where the C units are µg/mL and the t unit is days, derived from the original concentration assay and time data.

18.3.2.1 Calculating first order values from a linear rate equation

Examples—First order from a linear rate

1. Calculate the t_{90} expiration date:

$$\ln(C_0) = 4.382; \text{ thus, } C_0 = e^{4.382} = 80$$

$$\ln(0.9 \times 80) = 4.382 - 0.076(t_{90})$$

$$t_{90} = (4.277 - 4.382)/-0.076 = 1.4 \text{ days}$$

2. Calculate the time at which $C = 75$ µg/mL:

$$\ln(75) = 4.382 - 0.076t$$

General Chapters

$$t = (4.317 - 4.382)/-0.076 = 0.86 \text{ day}$$

3. Calculate whether $C = 70$ µg/mL occurs before or after t_{90}:

$$\ln(70) = 4.382 - 0.076t$$

$$t = (4.248 - 4.382)/-0.076 = 1.8 \text{ days}$$

$C = 70$ µg/mL occurs at 1.8 days, after a t_{90} of 1.4 days

18.3.3 FIRST ORDER EXPIRATION DATE CALCULATED FROM TWO VALUES OF CONCENTRATION AND TIME

When degradation or other cause of concentration loss is known from experience or reference information to obey first order kinetics, the rate constant can be accurately estimated from accurate assays of only two concentrations at their respective times. In this case, the linear first order rate equation, $\ln(C) = \ln(C_0) - kt$, may be transformed or integrated as $\ln(C_2) = \ln(C_1) - k(t_2 - t_1)$, which when rearranged is $k = \ln(C_1/C_2)/(t_2 - t_1)$. The following examples apply these equations to calculate expiration dates, concentrations, and times.

18.3.3.1 Calculating first order expiration date from two values

Examples—First order expiration date from two values

1. At 25°, the concentration of an antibiotic in solution was 89 mg/mL after 3 hours and 74 mg/mL after 8 hours. Calculate the initial concentration at time zero:

$$k = \ln(89/74)/(8 - 3) = 0.037 \text{ hour}^{-1}$$

$$\ln(89) = \ln(C_0) - (0.037 \text{ hour}^{-1} \times 3 \text{ hour})$$

$$\ln(C_0) = 4.489 + 0.111 = 4.6$$

$$C_0 = e^{4.6} = 99.5 \text{ mg/mL}$$

2. Calculate the t_{90} expiration date using the data in example 1. At t_{90}, $C = 0.9C_0$.

$$\ln(0.9 \times 99.5) = \ln(99.5) - 0.037(t_{90})$$

$$t_{90} = (4.495 - 4.600)/-0.037 \text{ hour}^{-1} = 2.8 \text{ hour}$$

3. Calculate the concentration at 6 hour using the data in example 1:

$$\ln(C) = \ln(99.5) - (0.037 \times 6)$$

$$\ln(C) = 4.378$$

$$C = e^{4.378} = 79.7 \text{ mg/mL}$$

18.3.4 FIRST ORDER TIMES, t_n, FOR 0.*n* FRACTION OR *n*% OF REMAINING ORIGINAL CONCENTRATION

The two most common first order pharmaceutical t_n values are the t_{50}, which is a primary parameter factor in clinical pharmacokinetics, and the t_{90}, which is the most common stability shelf life or expiration date. Values of any t_n, where $0 < n < 100$, are derived from the linear first order equation, $\ln(C) = \ln(C_0) - kt$. The equations for t_{50} and t_{90} in particular are derived in the following examples. The value of k by definition is constant for a specific drug chemical in a specific formulation at a specific temperature; thus, t_n values derived from such values of k are also constant.

18.3.4.1 Calculating first order times for remaining original concentrations

Examples—First order times for remaining original concentrations

1. At t_n, $C = 0.n \times C_0$.

$$\ln(0.n \times C_0) = \ln(C_0) - kt_n$$

$$t_n = [\ln(0.n \times C_0) - \ln(C_0)]/-k = \ln[(0.n \times C_0)/C_0]/-k = \ln(0.n)/-k$$

$$t_n = \ln(0.n)/-k$$

2. At t_{50}, $C = 0.5(C_0)$.

$$\ln(0.5 \times C_0) = \ln(C_0) - kt_{50}$$

$$t_{50} = [\ln(0.5 \times C_0) - \ln(C_0)]/-k = \ln[(0.5 \times C_0)/C_0]/-k = \ln(0.5)/-k = -0.693/-k = 0.693/k$$

$$t_{50} = 0.693/k$$

3. At t_{90}, $C = 0.9(C_0)$.

$$\ln(0.9 \times C_0) = \ln(C_0) - kt_{90}$$

$$t_{90} = [\ln(0.9 \times C_0) - \ln(C_0)]/-k = \ln[(0.9 \times C_0)/C_0]/-k = \ln(0.9)/-k = -0.105/-k = 0.105/k$$

$$t_{90} = 0.105/k$$

18.4 Stability Prediction Based on Arrhenius Theory

The basis of the Arrhenius theory is that reaction rates and rate constants change exponentially in the direction of arithmetic temperature change. The pharmaceutical application of the Arrhenius theory is based on scientifically accurate and statistically valid assay data obtained at three or more temperatures that are ≥10° warmer than the intended drug storage temperature and each other. The Arrhenius equation may be expressed in an exponential form, $k = Ae^{-(E_a/RT)}$, a linear form, $\ln(k) = \ln(A) - (E_a/RT)$, and an integrated form, $\ln(k_2/k_1) = E_a(T_2 - T_1)/[R(T_2 \times T_1)]$, where k, k_1, and k_2 are isothermal rate constants, A is a thermodynamic factor, E_a is energy of activation for the degradation reaction, R is the gas constant (1.987×10^{-3} kcal mol^{-1}K^{-1} or 8.314×10^{-3} J K^{-1} mol^{-1}), and T, T_1, and T_2 are absolute or Kelvin temperatures.

18.4.1 ARRHENIUS LINEAR EQUATION DERIVED FROM ORIGINAL DATA

The following examples illustrate derivation of a linear Arrhenius equation from original assay data and its application to predicting a drug stability expiration date at a cooler or lower storage temperature.

18.4.1.1 Calculating Arrhenius equations

Examples—Arrhenius equations

1. Calculate the linear Arrhenius equation based on the rate constants and temperatures for a beta-lactam antibiotic that decomposes in solution at a first order rate (see *Table 8*):

Table 8

T (°C)	T (K)	$1/T$ (K^{-1})	k (hour^{-1})	ln(k)
40	313	3.195×10^{-3}	0.0014	−6.571
50	323	3.096×10^{-3}	0.005	−5.298
60	333	3.003×10^{-3}	0.016	−4.135

Linear regression of the ln(k) (ordinate) *versus* $1/T$ (abscissa) values yields the equation, $\ln(k) = 33.977 - (12{,}689/T)$ with a correlation coefficient of 0.99997.

2. Calculate the t_{90} shelf life expiration date, in days, at 25°C (298 K) using the equation in example 1:

$$\ln(k_{25}) = 33.977 - (12{,}689/298) = 33.977 - 42.581 = -8.604$$

$$k_{25} = e^{-8.604} = 1.834 \times 10^{-4}\ \text{hour}^{-1} = 4.402 \times 10^{-3}\ \text{day}^{-1}$$

$$t_{90} = 0.105/k = 0.105/4.402 \times 10^{-3}\ \text{day}^{-1} = 23.85\ \text{days}$$

$$t_{90}\ \text{at}\ 25°\text{C} = 23.85\ \text{days}$$

18.4.2 STABILITY PREDICTIONS USING THE INTEGRATED ARRHENIUS EQUATION

The following examples illustrate stability predictions based on one accurately determined isothermal rate constant and adherence to the same degradation rate order, e.g., first order, at temperatures at which stability is to be calculated from the equation, $\ln(k_2/k_1) = E_a(T_2 - T_1)/[R(T_2 \times T_1)]$.

18.4.2.1 Calculating stability prediction using integrated Arrhenius equation

Example—Stability using integrated Arrhenius equations

1. Calculate the t_{85} stability expiration date at 4°C (277 K) for an ester hydrolysis with an E_a = 15 kcal/mol and k = 0.0045 hour^{-1} at 23°C (296 K):

$$\ln(k_{277}/0.0045) = 15(277 - 296)/[1.987 \times 10^{-3}\ (277 \times 296)]$$

$$\ln(k_{277}) - \ln(0.0045) = -285/162.92$$

$$\ln(k_{277}) = -1.749 + \ln(0.0045) = -7.153$$

$$k_{277} = e^{-7.153} = 7.825 \times 10^{-4}\ \text{hour}^{-1}$$

$$t_{85} = \ln(0.850)/k_{277} = -0.163/-7.825 \times 10^{-4}\ \text{hour}^{-1} = 208.3\ \text{hours}$$

$$t_{85}\ \text{at}\ 4°\text{C} = 208.3\ \text{hours}\ (t_{85}\ \text{at}\ 23°\text{C is}\ 36.2\ \text{hours})$$

18.4.3 ARRHENIUS-BASED Q_{10} STABILITY ESTIMATION

The temperature coefficient (Q_{10}) represents the multiplicative factor by which a chemical reaction rate constant changes in the same direction as the temperature for each 10°C change. For drug molecules, Q_{10} ranges from 2 to 5, corresponding to an E_a range of 10–25 kcal/mol or 42–105 kJ/mol. A Q_{10} of 3 yields reasonable estimates of drug stability in the equation, (t_n at T_2) = (t_n at T_1)/{$Q_{10}^{[(T_2-T_1)/10]}$}, where n is a percentage of remaining C_0, T_1 is the temperature at which t_n is known, and T_2 is the temperature at which t_n is to be estimated. Calculations using Q_{10} values of both 2 and 4 may be used to obtain the shortest or most conservative stability estimate, but Q_{10} = 3 is applied in the following two examples.

18.4.3.1 Calculating Arrhenius-based Q_{10} stability estimation

Example—Arrhenius-based Q_{10} stability

1. Estimate the t_{90} expiration date in hours of an antibiotic suspension stored in a closed automobile at 57° for which the 8° refrigeration t_{90} is 14 days.

$$t_{90} \text{ at } 57° = [14 \text{ days} \times (24 \text{ hours/day})]/\{3^{[(57-8)/10]}\} = 336 \text{ hours}/3^{4.9} = 336 \text{ hours}/217.7 = 1.54 \text{ hours}$$

$$t_{90} \text{ at } 57° = 1.54 \text{ hours}$$

APPENDIX 1: LOGARITHMS

The logarithm of a number is the exponent or power to which a given base number must be raised to equal that number. Thus, the logarithm of Y to the base, b, equals X, or $\log_b(Y) = X$. The logarithm of 0 and all negative numbers is undefined or nonexistent. The logarithm of 1 is 0 and of numbers <1 is negative in all systems (see *Table 9*).

Table 9. Common (or Briggsian) and Natural (or Napierian) Logarithms

Logarithmic System	Abbreviation or Symbol	Base Number	Format	Antilogarithm or Inverse Logarithm
Common	Log	10	$\log Y = X$	$10^X = Y$
Natural	Ln	e or 2.7183[a]	$\ln Y = X$	$e^X = Y$

[a] e is an irrational number derived from an infinite series of reciprocal whole number factorials, $e = 1 + 1/1! + 1/2! + 1/3! + 1/4! \ldots + 1/n!$, where n = infinity. e rounds to 2.7183 when $n \geq 8$.

2. The relationships between common and natural logarithms are the following:

(a) $\log Y = \ln Y/\ln 10 = \ln Y/2.303$

(b) $\ln Y = \ln 10 \times \log Y = 2.303 \times \log Y$

3. Rules for some common calculations with logarithms are shown in *Table 10*.

Table 10. Rules for Calculating with Logarithms

	Formula	Example
Additions and multiplications	$\ln(A) + \ln(B) = \ln(A \times B)$ $\log(A) + \log(B) = \log(A \times B)$	ln(0.62) + ln(1.73) = ln(0.62 × 1.73) = ln(1.0726) = 0.070 log(5.7) + log(0.43) = log (5.7 × 0.43) = log(2.451) = 0.389
Subtraction and quotients	$\ln(A) - \ln(B) = \ln(A/B)$ $\log(A) - \log(B) = \log(A/B)$	ln(0.5) – ln(4) = ln(0.5/4) = ln(0.125) = –2.079 log(1.57) – log(2.48) = log(1.57/2.48) = log(0.6330645) = –0.199
Simple non-base exponentials	$\ln(Y^Z) = Z \times \ln(Y)$ $\log(Y^Z) = Z \times \log(Y)$	$13.6^{-Z} = 1.25$ $\ln(1.25) = -Z \times \ln(13.6)$ $Z = -\ln(1.25)/\ln(13.6) = -0.223/2.610 = -0.085$ $0.57^Z = 2.3$ $\log(2.3) = Z \times \log(0.57)$ $Z = \log(2.3)/\log(0.57) = 0.362/-0.244 = -1.484$
Base exponentials	$\ln(a \times e^{\pm b}) = \ln(x) \leftrightarrow \ln(a) \pm b = \ln(x)$ $\log(a \times 10^{\pm b}) = \log(x) \leftrightarrow \log(a) \pm b = \log(x)$	$67 \times 10^b = 15.1$ $\log(67) + b = \log(15.1)$ $1.826 + b = 1.179$ $b = 1.179 - 1.826 = -0.647$

⟨1163⟩ QUALITY ASSURANCE IN PHARMACEUTICAL COMPOUNDING

INTRODUCTION

The need for a quality assurance system is well documented in *United States Pharmacopeia (USP)* chapters for compounded preparations (see *Quality Control* under *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Quality Assurance (QA) Program* under *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩). A quality assurance program is guided by written procedures that define responsibilities and practices that ensure compounded preparations are produced with quality attributes appropriate to meet the needs of patients and health care professionals. The authority and responsibility for the Quality Assurance program should be clearly defined and implemented and should include at least the following nine separate but integrated components: (1) training; (2) standard operating procedures (SOPs); (3) documentation; (4) verification; (5) testing; (6) cleaning, disinfecting, and safety; (7) containers, packaging, repackaging, labeling, and storage; (8) outsourcing, if used; and (9) responsible personnel.

The definition of compounding for the purpose of this chapter is defined in general test chapter ⟨795⟩.

The safety, quality, and efficacy and/or benefit of compounded preparations depend on correct ingredients and calculations; accurate and precise measurements; appropriate formulation, facilities, equipment, and procedures; and prudent pharmaceutical judgment. As a final check, the compounder shall review each procedure in the compounding process. To ensure accuracy and completeness, the compounder shall observe the finished preparation to ensure that it appears as expected and shall investigate any discrepancies and take appropriate corrective action before the prescription is dispensed to the patient.

The water used in all aspects of compounding should meet the requirements of *Waters for Pharmaceutical Purposes* ⟨1231⟩.

Radiopharmaceuticals and radiolabeled materials have unique characteristics requiring additional quality assurances described in *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses* ⟨823⟩ and the *Radiopharmaceuticals as CSPs* section under ⟨797⟩.

The responsibilities of the compounder and compounding personnel can be found in chapters ⟨795⟩ and ⟨797⟩.

TRAINING

Personnel involved in nonsterile or sterile compounding require additional, specific training and periodic retraining beyond the training needed for routine dispensing duties. A thorough quality assurance program for compounded preparations requires documentation of both training and skill competency. In addition, the authority and responsibility for the QA program should be clearly defined as implemented. Training for nonsterile compounders should meet or exceed the standards set forth in ⟨795⟩, and personnel training for sterile preparation compounders should meet or exceed the standards set forth in ⟨797⟩.

STANDARD OPERATING PROCEDURES

SOPs for pharmaceutical compounding are documents that describe how to perform routine and expected tasks in the compounding environment, including but not limited to procedures involving:

- Beyond-Use dating
- Chemical and physical stability
- Cleaning and disinfecting
- Component quality evaluation
- Compounding methods
- Dispensing
- Documentation
- Environmental quality and maintenance
- Equipment maintenance, calibration, and operation
- Formulation development
- Labeling
- Materials and final compounded preparation handling and storage
- Measuring and weighing
- Packaging and repackaging
- Patient monitoring, complaints, and adverse event reporting
- Patient or caregiver education and training
- Personnel cleanliness and garb
- Purchasing
- Quality Assurance and Continuous Quality Monitoring
- Safety
- Shipping
- Testing

- Training and retraining

SOPs are itemized instructions that describe when a task will be performed, how a task will be performed, who will perform the task, why the task is necessary, any limitations in performing the task, and what action to take when unacceptable deviations or discrepancies occur.

SOPs must be reviewed regularly and updated as necessary. Auditing and verifying compliance with established SOPs should be performed periodically. The SOP should be specific to each device and process used in compounding. Properly maintained and implemented SOPs are vital to preparation quality.

DOCUMENTATION

The purpose of documentation is to provide a record of all aspects of compounding operations and procedures that are described in this chapter, in 〈795〉, and in 〈797〉. Information on the compounding record should ideally be entered as the tasks are performed or as testing data is received. Compounding records should be reviewed for accuracy, completeness (as appropriate) and approved by QA personnel, prior to dispensing. Additionally, beyond-use dating and sterility studies, where appropriate, should be documented by reference to at least one of the following:

- Stability studies published in peer-reviewed literature,
- In-house or laboratory conducted stability and/or sterility studies,
- National compendia, or
- An extrapolation of above based on professional judgment.

VERIFICATION

Verification involves authoritatively signed assurance and documentation that a process, procedure, or piece of equipment is functioning properly and producing the expected results. The act of verification of a compounding procedure involves checking to ensure the calculations, weighing and measuring, order of mixing, and compounding techniques and equipment were appropriate and accurately performed. The quality of ingredients should be verified upon receipt (e.g., Certificate of Analysis, manufacturer's label on commmercial products, etc.). Verification may require outside laboratory testing when in-house capabilities are not adequate. Equipment verification methods are sometimes available from manufacturers of the specific equipment or can be developed in-house. The responsibility for assuring that equipment performance is verified, including work completed by contractors, resides with the compounder.

See *Component Selection, Handling, and Storage* under 〈795〉.

TESTING

A quality assurance program for compounded preparations should include testing during the compounding process and of the finished compounded preparation, when appropriate, as described in chapters 〈795〉 and 〈797〉. The compounder should have a basic understanding of pharmaceutical analysis to ensure that valid results are obtained when tests are being conducted, whether they are done in-house or outsourced. Acceptance criteria shall be determined prior to testing. Testing every compounded preparation is neither practical nor officially required, but compounders should conduct visual inspections and know: (1) the importance of testing in the overall quality program in the compounding facility, (2) when to test, (3) what to test, (4) what appropriate method(s) and equipment to use, (5) how to interpret the results, (6) the limits of the test, and (7) specific actions required when a preparation does not meet specifications. Investigative and corrective action should extend to other preparations that may have been associated with the specific failure or discrepancy. Testing may involve one or more quality attributes, and each test will have one or more acceptable procedures, usually with well-defined acceptance criteria.

The goal in testing is to determine accurately the adequacy of the compounding process and the quality of the preparation. Any testing procedure used should have accuracy, reproducibility, and specificity. No single testing procedure is suitable for all drugs or preparations because a number of factors determine the validity and reliability of results.

Compounding professionals have two options for the testing that is required for compounded preparations or their ingredients. Some testing methods can easily be performed at the compounding site, but some may need to be outsourced to a contract laboratory. Some testing methods can be conducted in-house by an individual who possesses a good understanding of pharmaceutical analysis and proper training. See *Table 1* for a list of compendial testing methods and *USP* chapters for reference.

Table 1. U.S. Pharmacopeia Chapters for Selected Quality Testing Methods and Procedures

	Chapter Title	Chapter
General Testing		
Boiling point	Distilling Range	〈721〉
Density	Density of Solids	〈699〉
Ion selective potentiometry	—	—

General Chapters

Table 1. U.S. Pharmacopeia Chapters for Selected Quality Testing Methods and Procedures *(Continued)*

	Chapter Title	Chapter
Loss on drying	Loss on Drying Pharmaceutical Calculations in Prescription Compounding	〈731〉 〈1160〉
Melting point	Melting Range or Temperature	〈741〉
Osmolality and osmolarity	Pharmaceutical Calculations in Prescription Compounding Osmolality and Osmolarity	〈1160〉 〈785〉
Particle size	Powder Fineness	〈811〉
Particulate matter in injections	Particulate Matter in Injections	〈788〉
pH	pH	〈791〉
Refractive index	Refractive Index	〈831〉
Viscosity change	Viscosity—Capillary Methods	〈911〉
Volumetric	Prescription Balances and Volumetric Apparatus	〈1176〉
Weight	Prescription Balances and Volumetric Apparatus	〈1176〉
Spectroscopy		
Atomic absorption spectroscopy	Atomic Absorption Spectroscopy	〈852〉
Fluorescence spectroscopy	Fluorescence Spectroscopy	〈853〉
Mid-Infrared spectroscopy	Mid-Infrared Spectroscopy	〈854〉
Ultraviolet/visible spectroscopy	Ultraviolet-Visible Spectroscopy	〈857〉
Chromatography		
Column chromatography (CC)	Chromatography	〈621〉
Gas chromatography (GC)	Chromatography	〈621〉
High-performance liquid chromatography (HPLC)	Chromatography	〈621〉
Paper chromatography (PC)	Chromatography	〈621〉
Thin-layer chromatography (TLC)	Chromatography	〈621〉
Microbiology		
Endotoxin testing	Bacterial Endotoxins Test	〈85〉
Microbial limit testing	Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms	〈61〉 〈62〉
Preservative effectiveness testing	Antimicrobial Effectiveness Testing	〈51〉
Sterility	Sterility Tests	〈71〉

If testing is done at the compounding site, appropriate equipment shall be obtained and qualified either by the manufacturer upon sale or by the compounding professional upon receipt and shall be maintained, calibrated, and used properly. If testing is outsourced, the compounding professional should determine what to outsource, how to select a laboratory, and should develop an ongoing relationship with the laboratories chosen. Contract laboratories shall follow standards set forth in *USP* general chapters, as appropriate, and preferably should be registered with the U.S. Food and Drug Administration (FDA).

Selection of a Testing Method

One general consideration in testing procedure selection is the type of information needed, such as quantitative (strength, concentration), semiquantitative (where a tolerance level is involved, as in endotoxin levels), or qualitative (presence/absence testing, including substance identification, sterility). Another consideration involves the physical and chemical characteristics of the analyte, including solubility, partition coefficient, dissociation constant (pKa), volatility, binding, and the quantity present. The testing method selected also depends upon factors such as sample handling/preparation/purification requirements; type of data needed; and accuracy, reproducibility, and specificity required.

The degree of quantitative measurement and specificity must be considered in the verification process. The typical analytical characteristics used in method verification include accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and ruggedness. Generally, the greater the level of accuracy, precision, or specificity required, the more sophisticated and expensive the testing methods needed. The methods used are also governed by the types of instrumentation available and the standards available for comparison.

Pharmaceutical analysis decisions include procedure selection, obtaining a representative sample (the number of preparation units selected to adequately represent the entire formulation, e.g., 10 randomly selected capsules from a preparation of 100

General Chapters

capsules), storage/shipping of the sample, sample preparation for analysis, the actual analysis, data acquisition, data treatment, and interpretation.

The compounding professional is responsible for implementing a program using selected testing methods for the preparations compounded in the facility. *USP* chapters on spectroscopy and chromatography methods are referenced in *Table 1*. Examples of general and microbiological testing methods are discussed later in this chapter. Examples of selected testing methods for bulk substances and various dosage forms (see *Pharmaceutical Dosage Forms* ⟨1151⟩) are shown in *Table 2*.

Table 2. Selected Compendial Testing Methods for Bulk Substances and Various Dosage Forms

Bulk Substances and Dosage Forms	Testing Method[a]													
	Wt	Vol	pH	Osm	RI	Sp Gr	MP	UV/Vis	HPLC	GC	IR	Sterile	Endotoxin	PM
Bulk substances	–	–	+	–	+	–	+	+	+	+	+	–	+[b]	–
Capsules	+	–	–	–	–	–	–	–	+	+	–	–	–	–
Emulsions	+	+	+	–	–	+	–	–	+	+	–	–	–	–
Gels	+	+	+	–	+	+	–	–	+	+	–	–	–	–
Inhalations	+	+	+	+	+	+	–	+	+	+	–	+	+	–
Injections	+	+	+	+	+	+	–	+	+	+	–	+	+	+
Inserts	+	–	–	–	–	+	+	–	+	+	–	–	–	–
Irrigations	+	+	+	+	+	+	–	+	+	+	–	+	+	–
Lozenges	+	–	–	–	–	–	–	–	+	+	–	–	–	–
Nasals	+	+	+	+	+	+	–	+	+	+	–	*[c]	–	–
Ophthalmics	+	+	+	+	+	+	–	+	+	+	–	+	–	+[d]
Otics	+	+	+	+	+	+	–	+	+	+	–	–	–	–
Powders	+	–	–	–	–	–	–	–	+	+	–	–	–	–
Semisolids	+	–	+	–	–	+	+	–	+	+	–	–	–	–
Solutions, nonsterile	+	+	+	+	+	+	–	+	+	+	–	–	–	–
Sterile implant gels	+	+	+	+	+	+	–	+	+	+	–	+	+	–
Sterile implant solids	+	+	–	–	–	–	+	+	+	+	–	+	+	–
Sticks	+	–	–	–	–	+	+	–	+	+	–	–	–	–
Suppositories	+	–	–	–	–	+	+	–	+	+	–	–	–	–
Suspensions, nonsterile	+	+	+	–	–	+	–	–	+	+	–	–	–	–
Tablets	+	–	–	–	–	–	–	–	+	+	–	–	–	–

[a] Wt, weight; Vol, volume; Osm, osmolality/osmolarity; RI, refractive index; Sp Gr, specific gravity; MP, melting point; UV/Vis, ultraviolet/visible spectroscopy; HPLC, high-performance liquid chromatography; GC, gas chromatography; IR, infrared spectroscopy; PM, particulate matter; +, test applicable; –, test not applicable.

[b] Endotoxin testing may be needed for bulk substances used in compounding some sterile preparations.

[c] *, microbial limits (see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩).

[d] Solutions only, not suspensions or ointments.

Sampling Requirements

Before collecting samples for testing, compounding professionals should consider the following factors:

- Quantity of preparation being compounded, for a specific prescription versus in anticipation of prescriptions routinely received
- Number of samples needed
- Destructive or nondestructive testing
- Appropriate methods of obtaining representative samples
- Physical state of the samples (solid, liquid, or gas)
- Type of container required for collection and storage
- Any special handling and shipping requirements or restrictions (e.g., controlled drug substances, dangerous or hazardous chemicals, flammable or caustic substances, and refrigerated or frozen preparations)

Storage Requirements

Storage requirements for samples must be specified, including type of container, temperature, humidity, and light protection (see *General Notices and Requirements* and the *Containers, Packaging, Repackaging, Labeling, and Storage* section in this chapter).

The effect(s) that any substance has on the compounded preparation that may interfere or alter the results must be known beforehand. When sending a preparation to a contract laboratory, the compounder should provide the complete written formulation so that the laboratory can quickly determine if there may be any interfering substances present.

Data Interpretation Requirements

The collection of raw data from the testing process must be completed accurately. One must ensure that appropriate and valid descriptive statistics (e.g., mean, standard deviation) are used to analyze the data and that the operating parameters of the analytical instruments are well-established. Reference values, if available, should be provided with the analytical results. A description of the analytical controls used by the laboratory is important for documentation, as is the source of reference standards used to establish standard curves.

Personnel Requirements and Considerations

If testing is done in-house, personnel involved in this activity must be appropriately trained and evaluated with documentation of the training and evaluation. If testing is outsourced, the compounder must be assured of the credentials, proper training, and continuing competency activities of the personnel in the contract laboratory.

PHYSICAL TESTING OF DOSAGE UNITS

[NOTE—In this section the terms "unit" and "dosage unit" are synonymous. To ensure the consistency of dosage units, each unit in a batch should have a uniform weight within a narrow range. Dosage units are defined as dosage forms containing a single dose or a part of a dose in each unit. If multiple dose units are compounded in a batch formulation, the total number of units should not deviate outside of ±10% of the theoretical number of units.]

WEIGHT ASSESSMENT

First, zero or tare the balance. During the compounding process intermediate weighing may be necessary to ensure that all substances have been included and weighed accurately.

At the end of the compounding process, for the dosage form and quantity designated, take care to preserve the integrity of each dosage unit during the following assessment procedures. Assume the concentration (weight of drug substance per weight of dosage unit) is uniform. The following are examples of weight assessment.

Hard Capsules

- Zero or tare balance with an empty capsule.
- Accurately weigh each individual filled capsule from a representative sample of the finished batch (for example, a minimum of 5% of total capsules or 10 individual capsules, whichever is less) and record the weight of each finished capsule on the compounding record.
- Calculate the theoretical weight of a finished capsule's contents.
- Compare the actual content weight of each finished capsule in the representative sample with the theoretical weight of a finished capsule's contents.
- Determine if there is a deviation outside ±10% with any weight of a finished capsule's contents and the theoretical weight of a finished capsule, and if so,
  - Review the compounding record to ensure no steps were omitted.
  - Repeat with a larger representative sample of the finished batch (10% of total capsules or 20 individual capsules, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of ±10% is discovered in the second representative sample, then destroy the batch.

Other Solids (Including Tablets, Suppositories, Inserts, and Lozenges)

- Accurately weigh each individual dosage unit from a representative sample of the finished batch (for example, a minimum of 5% of total tablets or 10 individual tablets, whichever is less) and record the weight of each dosage unit on the compounding record.
- Calculate the theoretical weight of the dosage unit.
- Compare the actual weight of each dosage unit in the representative sample with the theoretical weight of a dosage unit.
- Determine if there is a deviation outside ±10% with any weight of a finished dosage unit and the theoretical weight of a finished dosage unit, and if so,
  - Review the compounding record to ensure no steps were omitted.

General Chapters

— Repeat with a larger representative sample of the finished batch (10% of total tablets or 20 individual tablets, whichever is less). Do not mix with the first batch tested.

- If a deviation outside of ±10% is discovered in the second representative sample, then destroy the batch.

Semi-Solids (Including Creams, Gels, and Ointments)

- Accurately weigh an empty container and record the weight on the compounding record.
- Fill an empty container with the final compounded preparation.
- Calculate the theoretical weight of the compounded preparation.
- Weigh the filled container.
- Determine if there is a deviation outside of ±10%, and if so, review the compounding record to ensure no steps were omitted. If the deviation cannot be explained, destroy the batch and prepare a new one.

Additional Quality Assurance Checks Before Packaging Semi-Solids—

— Visually inspect the preparation for foreign materials and expected appearance.

— Measure pH, when applicable.

MICROBIOLOGICAL TESTING

Microbiological testing for pharmacy compounding includes sterility, endotoxin, preservative effectiveness testing, and microbial limit testing (see ⟨797⟩).

Sterility Testing

Sterility tests may be conducted using commercial kits or by developing and verifying USP sterility testing protocols. Standards and procedures are explained in ⟨71⟩.

Endotoxin Testing

Endotoxin tests may be conducted using commercial kits or by purchasing the components separately. Endotoxin testing may be performed in-house with appropriate training and experience. See ⟨85⟩.

Preservative Effectiveness Testing

Preservative effectiveness testing may be conducted when preparing a frequently compounded formulation that contains a preservative. When such a test is performed, the results shall support the beyond-use-date (BUD) assigned to the compounded preparations. See ⟨51⟩.

Microbial Limit Testing

Microbial limit testing may be conducted to provide an estimate of the number of viable aerobic microorganisms (see ⟨61⟩) or to demonstrate freedom from designated microbial species (see ⟨62⟩).

CLEANING, DISINFECTING, AND SAFETY

This section applies to both equipment and facilities (see ⟨795⟩, ⟨797⟩, and *Disinfectants and Antiseptics* ⟨1072⟩).

CONTAINERS, PACKAGING, REPACKAGING, LABELING, AND STORAGE

For storage, packaging, repackaging, and labeling of compounded preparations and repackaging of manufactured products (when defined as compounding in *USP*), refer to *USP General Notices and Requirements* and the following general chapters:

- *Containers—Glass* ⟨660⟩
- *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩
- *Plastic Materials of Construction* ⟨661.1⟩
- *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩
- *Elastomeric Closures for Injections* ⟨381⟩
- *Good Packaging Practices* ⟨1177⟩
- *Good Repackaging Practices* ⟨1178⟩
- *Good Storage and Distribution Practices for Drug Products* ⟨1079⟩
- *Labeling* ⟨7⟩
- *Packaging and Storage Requirements* ⟨659⟩

- *Packaging and Repackaging—Single-Unit Containers* ⟨1136⟩
- *Pharmaceutical Dosage Forms* ⟨1151⟩

OUTSOURCING

[NOTE—This section addresses only the purchasing or selling of compounded preparations from pharmacy to pharmacy, not the outsourcing of analytical testing of compounded preparations.]

For pharmacies that prepare outsourced compounded preparations or repackaged commercial products, documentation of beyond-use dating, as defined previously in the *Documentation* section of this chapter, is required and shall be provided upon request. In addition, documentation of compliance with *USP* chapters ⟨795⟩ and ⟨797⟩ is required and shall be provided upon request.

For facilities that receive outsourced compounded preparations or repackaged commercial products, documentation shall be on file for all BUDs assigned to those preparations or products.

RESPONSIBLE PERSONNEL

The responsibility and authority for a quality assurance program should be clearly defined and implemented. Personnel responsible for the quality assurance program should have the education, training, and experience necessary to perform the assigned functions. Quality assurance personnel should assure that documentation, verification, and testing are performed in accordance with written policies and procedures. If deviations from approved policies and procedures occur, it is the responsibility of the quality assurance personnel to investigate and to implement appropriate corrective action. Documentation of any investigations and corrective actions is the responsibility of the quality assurance personnel. Responsible personnel in the quality assurance program are essential in assuring the safety, identity, strength, quality, and purity of compounded drug preparations.

SUMMARY

A quality assurance program is necessary to ensure the quality of compounded preparations. A sound quality assurance program includes detailed SOPs, documentation, verification, analytical and microbiological testing as appropriate to particular compounded preparations, and responsible quality assurance personnel. Compounding professionals must determine the types of testing and degree of testing that will be a part of their quality assurance program. They also must decide whether to perform testing in-house or outsource it to a contract laboratory.

⟨1171⟩ PHASE-SOLUBILITY ANALYSIS

Phase-solubility analysis is the quantitative determination of the purity of a substance through the application of precise solubility measurements. At a given temperature, a definite amount of a pure substance is soluble in a definite quantity of solvent. The resulting solution is saturated with respect to the particular substance, but the solution remains unsaturated with respect to other substances, even though such substances may be closely related in chemical structure and physical properties to the particular substance being tested. Constancy of solubility, like constancy of melting temperature or other physical properties, indicates that a material is pure or is free from foreign admixture except in the unique case in which the percentage composition of the substance under test is in direct ratio to solubilities of the respective components. Conversely, variability of solubility indicates the presence of an impurity or impurities.

Phase-solubility analysis is applicable to all species of compounds that are crystalline solids and that form stable solutions. It is not readily applicable to compounds that form solid solutions with impurities.

The standard solubility method consists of six distinct steps: (1) mixing, in a series of separate systems, increasing quantities of material with measured, fixed amounts of a solvent; (2) establishment of equilibrium for each system at identical constant temperature and pressure; (3) separation of the solid phase from the solutions; (4) determination of the concentration of the material dissolved in the various solutions; (5) plotting the concentration of the dissolved materials per unit of solvent (y-axis or solution composition) against the weight of material per unit of solvent (x-axis or system composition); and (6) extrapolation and calculation.

SOLVENTS

A proper solvent for phase-solubility analysis meets the following criteria: (1) The solvent is of sufficient volatility that it can be evaporated under vacuum, but is not so volatile that difficulty is experienced in transferring and weighing the solvent and its solutions. Normally, solvents having boiling points between 60° and 150° are suitable. (2) The solvent does not adversely

affect the substance being tested. Solvents that cause decomposition or react with the test substance are not to be used. Solvents that solvate or form salts are to be avoided, if possible. (3) The solvent is of known purity and composition. Carefully prepared mixed solvents are permissible. Trace impurities may affect solubility greatly. (4) A solubility of 10 mg to 20 mg per g is optimal, but a wider working range can be used.

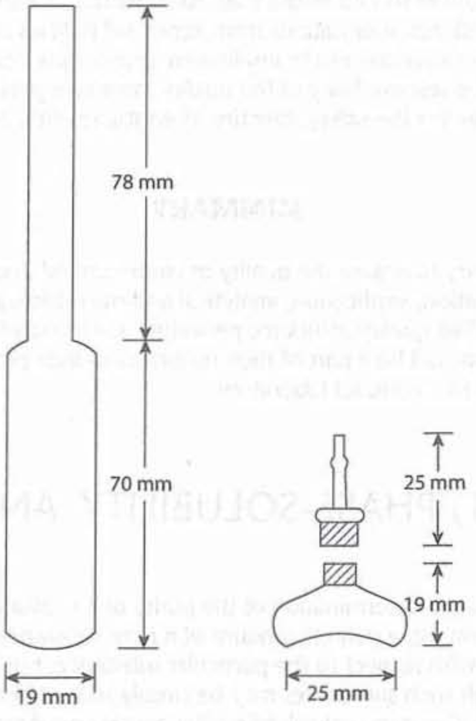
APPARATUS*

Constant-Temperature Bath

Use a constant-temperature bath that is capable of maintaining the temperature within ±0.1° and that is equipped with a horizontal shaft capable of rotating at approximately 25 rpm. The shaft is equipped with clamps to hold the *Ampuls*. Alternatively, the bath may contain a suitable vibrator, capable of agitating the ampuls at 100 to 120 vibrations per second, and equipped with a shaft and suitable clamps to hold the ampuls.

Ampuls

Use 15-mL ampuls of the type shown in the *accompanying illustration*. Other containers may be used provided that they are leakproof and otherwise suitable.



Ampul (left) and Solubility Flask (right) Used in Phase-Solubility Analysis

Solubility Flasks

Use solubility flasks of the type shown in the *accompanying illustration*.

PROCEDURE

NOTE—Make all weighings within ±10 µg.

System Composition

Weigh accurately, in g, not less than 7 scrupulously cleaned 15-mL ampuls. Weigh accurately, in g, increasingly larger amounts of the test substance into each of the ampuls. The weight of the test substance is selected so that the first ampul contains slightly less material than will go into solution in 5 mL of the selected solvent, the second ampul contains slightly

* Available from Hanson Research Corp., 19727 Bahama St., P. O. Box 35, Northridge, CA 91324.

more material, and each subsequent ampul contains increasingly more material than meets the indicated solubility. Transfer 5.0 mL of the solvent to each of the ampuls, cool in a dry ice–acetone mixture, and seal, using a double-jet air-gas burner and taking care to save all glass. Allow the ampuls and their contents to come to room temperature, and weigh the individual sealed ampuls with the corresponding glass fragments. Calculate the system composition, in mg per g, for each ampul by the formula:

$$1000(W_2 - W_1)/(W_3 - W_2)$$

in which W_2 is the weight of the ampul plus test substance, W_1 is the weight of the empty ampul, and W_3 is the weight of ampul plus test substance, solvent, and separated glass.

Equilibration

The time required for equilibration varies with the substance, the method of mixing (rotation or vibration), and the temperature. Normally, equilibrium is obtained more rapidly by the vibration method (1 to 7 days) than by the rotational method (7 to 14 days). In order to determine whether equilibration has been effected, 1 ampul, i.e., the next to the last in the series, may be warmed to 40° to produce a supersaturated solution. Equilibration is ensured if the solubility obtained on the supersaturated solution falls in line with the test specimens that approach equilibrium from an undersaturated solution.

Solution Composition

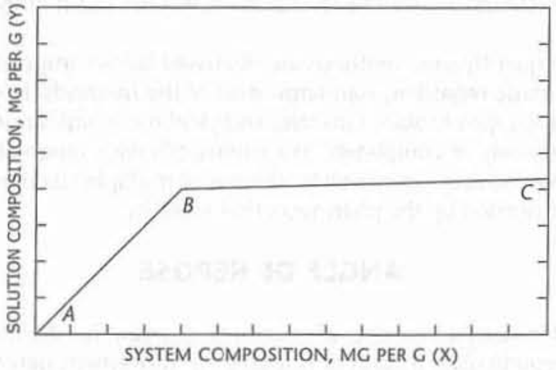
After equilibration, place the ampuls vertically in a rack in the constant-temperature bath, with the necks of the ampuls above the water level, and allow the contents to settle. Open the ampuls, and remove a portion greater than 2 mL from each by means of a pipet equipped with a small pledget of cotton membrane or other suitable filter. Transfer a 2.0-mL aliquot of clear solution from each ampul to a marked, tared solubility flask, and weigh each flask plus its solution to obtain the weight of the solution. Cool the flasks in a dry ice–acetone bath, and then evaporate the solvent in vacuum. Gradually increase the temperature to a temperature consistent with the stability of the compound, and dry the residue to constant weight. Calculate the solution composition, in mg per g, by the formula:

$$1000(F_3 - F_1)/(F_2 - F_3)$$

in which F_3 is the weight of the flask plus residue, F_1 is the weight of the solubility flask, and F_2 is the weight of the flask plus solution.

CALCULATION

For each portion of the test substance taken, plot the solution composition as the ordinate and the system composition as the abscissa. As shown in the *accompanying diagram,*



Typical Phase-Solubility Diagram

the points for those containers, frequently only one, that represent a true solution fall on a straight line (AB) with a slope of 1, passing through the origin; the points corresponding to saturated solutions fall on another straight line (BC), the slope, S, of which represents the weight fraction of impurity or impurities present in the test substance. Failure of points to fall on a straight line indicates that equilibrium has not been achieved. A curve indicates that the material under test may be a solid solution. Calculate the percentage purity of the test substance by the formula:

$$100 - 100S.$$

General Chapters

The slope, S, may be calculated graphically or by least-squares treatment for best fit of the experimental values to a straight line.

The solubility of the main component is obtained by extending the solubility line (BC) through the y-axis. The point of interception on the y-axis is the extrapolated solubility, in mg per g, and is a constant for a given compound.

PURIFICATION TECHNIQUE

Since the solvent phase in all combinations of solvent and solute that are used to construct segment BC of a phase-solubility diagram contains essentially all the impurities originally present in the substance under analysis, whereas the solid phase is essentially free from impurities, phase-solubility analysis can be used to prepare pure reference specimens of desired compounds as well as concentrates of impurities from substances otherwise considered pure. A simple modification of this technique can be used to accomplish these purposes with considerably less effort than is usually required for rigorous phase-solubility analysis.

In practice, a weighed amount of test specimen is suspended in a nonreactive solvent of suitable composition and amount so that about 10% of the material is dissolved at equilibrium. The suspension is sealed (a screw-cap vial is usually adequate) and shaken at room temperature until equilibrium is attained (usually 24 hours is sufficient for this purpose). The mother liquor is then drawn off and evaporated at or near room temperature to dryness. Since the mother liquor contained essentially all the impurities that were present in the specimen, the residue has been concentrated with respect to the impurities roughly in proportion to the ratio of the weight of specimen taken to the weight of solids dissolved in the volume of solvent used.

The undissolved crystals remaining after withdrawal of the mother liquor are usually sufficiently pure to be used as a reference standard after appropriate rinsing and drying.

⟨1174⟩ POWDER FLOW

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index or Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise as a result of segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

Basic Methods for Angle of Repose

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified on the basis of the following two important experimental variables:

(1) The height of the "funnel" through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.

(2) The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

Variations in Angle of Repose Methods

In addition to the above methods, the following variations have been used to some extent in the pharmaceutical literature:

- *Drained angle of repose* is determined by allowing an excess quantity of material positioned above a fixed diameter base to "drain" from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.
- *Dynamic angle of repose* is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

Angle of Repose General Scale of Flowability

Although there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr*, which is shown in *Table 1*. There are examples in the literature of formulations with an angle of repose in the range of 40° to 50° that were manufactured satisfactorily. When the angle of repose exceeds 50°, the flow is rarely acceptable for manufacturing purposes.

Table 1. Flow Properties and Corresponding Angles of Repose*

Flow Property	Angle of Repose (degrees)
Excellent	25–30
Good	31–35
Fair—aid not needed	36–40
Passable—may hang up	41–45
Poor—must agitate, vibrate	46–55
Very poor	56–65
Very, very poor	>66

Experimental Considerations for Angle of Repose

Angle of repose is not an intrinsic property of the powder; i.e., it is very much dependent upon the method used to form the cone of powder. The following important considerations are raised in the existing literature:

- The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.
- The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a "common base," which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

Recommended Procedure for Angle of Repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2–4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α, from the following equation:

$$\tan(\alpha) = \text{height}/0.5\ \text{base}$$

* Carr, R.L. Evaluating Flow Properties of Solids. *Chem. Eng.* **1965**, *72*, 163–168.

General Chapters

COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and the tapped volume of a powder.

Basic Methods for Compressibility Index and Hausner Ratio

Although there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume, V_O, and (2) the final tapped volume, V_f, of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{Compressibility Index} = 100 \times [(V_O - V_f)/V_O]$$

$$\text{Hausner Ratio} = (V_O/V_f)$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\text{Compressibility Index} = 100 \times [(\rho_{tapped} - \rho_{bulk})/\rho_{tapped}]$$

$$\text{Hausner Ratio} = (\rho_{tapped}/\rho_{bulk})$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in *Table 2**.

Table 2. Scale of Flowability*

Compressibility Index (%)	Flow Character	Hausner Ratio
≤10	Excellent	1.00–1.11
11–15	Good	1.12–1.18
16–20	Fair	1.19–1.25
21–25	Passable	1.26–1.34
26–31	Poor	1.35–1.45
32–37	Very poor	1.46–1.59
>38	Very, very poor	>1.60

Experimental Considerations for the Compressibility Index and Hausner Ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder; i.e., they depend on the methodology used. In the existing literature, there are discussions of the following important considerations affecting the determination of (1) the unsettled apparent volume, V_o, (2) the final tapped volume, V_f, (3) the bulk density, ρ_{bulk}, and (4) the tapped density, ρ_{tapped}:

- The diameter of the cylinder used
- The number of times the powder is tapped to achieve the tapped density
- The mass of material used in the test
- Rotation of the sample during tapping

Recommended Procedure for Compressibility Index and Hausner Ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously because pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

Basic Methods for Flow Through an Orifice

There are a variety of methods described in the literature. The most common method for determining the flow rate through an orifice can be classified on the basis of three important experimental variables:

(1) The type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment.

(2) The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.

(3) The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

Variations in Methods for Flow Through an Orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Because die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container; however, this appears to complicate interpretation of the results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

General Scale of Flowability for Flow Through an Orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

Experimental Considerations for Flow Through an Orifice

Flow rate through an orifice is not an intrinsic property of the powder. It very much depends on the methodology used. Several important considerations affecting these methods are discussed in the existing literature:

- The diameter and shape of the orifice
- The type of container material (metal, glass, plastic)
- The diameter and height of the powder bed.

Recommended Procedure for Flow Through an Orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the "head" of the powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- Diameter of opening > 6 times the diameter of the particles
- Diameter of the cylinder > 2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, making selection of an appropriate construction material an important consideration.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and to better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

Basic Methods for Shear Cell

One type of shear cell is the cylindrical shear cell that is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly; i.e., material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology is rather time-consuming and requires significant amounts of material and a well-trained operator.

Recommendations for Shear Cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

⟨1176⟩ PRESCRIPTION BALANCES AND VOLUMETRIC APPARATUS USED IN COMPOUNDING

1. BALANCES
- 1.1 Mechanical Prescription Balances
- 1.2 Electronic Balances
- 1.3 Minimum Accurately Weighable Quantity on the Balance

2. VOLUMETRIC APPARATUS
- 2.1 Selection and Use of Graduates
- 2.2 Medicine Droppers
- 2.3 Dispensing Bottles
- 2.4 Syringes
- 2.5 Pipets
- 2.6 Volumetric Flasks
- 2.7 Additional Considerations When Using Volumetric Apparatus

1. BALANCES

Pharmacies that perform materials measurements for compounding and dispensing should have access to a well-maintained National Institute of Standards and Technology (NIST) Class III torsion prescription balance or superior balance (preferably an electronic balance) to weigh masses accurately. The pharmacy should have a set of calibration weights, or the balance should have internal calibration capability to standardize the precision and accuracy of the balance.

For more information regarding standards for weights and balances, see *Balances* ⟨41⟩. The standards in NIST Handbook 44 called "Specifications, Tolerances, and Other Technical Requirements for Weighing and Measuring Devices" may also be useful to compounders.[1] Some balances offer digital or direct-reading features and printing capabilities, which may be desirable for ease of use. All balances should be calibrated or verified and tested frequently using appropriate test weights.

1.1 Mechanical Prescription Balances

A Class III (formerly Class A) prescription balance is a device designed to accurately weigh drugs and other substances and materials required in pharmaceutical compounding and dispensing practice.[1] It is constructed to support its full weighing capacity without developing undue stresses, and its adjustment is not altered by repeated weighing of the capacity load. The removable pans or weighing vessels should be of equal weight and shape. The balance should have leveling feet or screws and a built-in means to observe its level. The balance may feature dial-in weights and also a precision spring and dial instead of a weighbeam. A balance that has a graduated weighbeam must have a stop that halts the rider or poise at the zero reading, and the reading edge of the rider must be parallel to the graduations on the weighbeam. The distance from the face of the index plate to the indicator pointer or pointers should be NMT 1.0 mm; the points should be sharp; and when there are two pointers, the ends should be separated by NMT 1.0 mm when the scale is in balance. The indicating components and the lever system should be protected against drafts; the balance lid should permit free movement of the loaded weighing pans when the lid is closed; and the balance must have a mechanical arresting device.

BALANCE TESTING PROCEDURE

A Class III prescription balance meets the criteria in the following four tests using an ANSI/ASTM E617 set of Class 6 or better test weights. A typical apothecary weight set contains weights ranging from 10 mg to 50 g in sizes of 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 1 g, 2 g, 5 g, 10 g, 20 g, and 50 g.

Four tests are required to evaluate the suitability of a Class III prescription balance. A balance is acceptable if it passes all four tests. If the balance fails any test, it should not be used until it has been repaired to meet specifications and retested. The four tests are:

1. Sensitivity requirement
2. Arm ratio test
3. Shift test
4. Rider and graduated beam

Sensitivity requirement test: The following requirements are based on (1) the NIST standard that a 6-mg load must displace the pointer 2 mm,[1] and (2) the absence of a 6-mg weight in a typical apothecary weight set.

[1] NIST Handbook 44 (2015) Specifications, Tolerances, and Other Technical Requirements for Weighing and Measuring Devices. Section 2.2.0 Scales (http://www.nist.gov/pml/wmd/pubs/h44-11.cfm).

a. For balances with pointer scale division marks 2 mm apart, a 10-mg weight on one pan and no weight on the other pan, dial, or rider beam of a leveled balance should displace the pointer 1.5-scale division marks, or approximately 6.7 mg/mark, as observed visually. This same 1.5-scale division mark displacement shall be observed with a 10-g weight on one pan and 10-g plus 10-mg weights on the other pan of a level balance.

b. For balances on which the pointer scale division marks are 1 mm apart, a 10-mg weight on one pan and no weight on the other pan, dial, or rider beam of a leveled balance should displace the pointer 3-scale division marks, or approximately 6.7 mg/two marks, as observed visually. This same 3-scale division marks displacement shall be observed with a 10-g weight on one pan and 10-g plus 10-mg weights on the other pan of a level balance.

c. NLT 0.12 g (120 mg) shall be weighed on a Class III prescription to reduce reading errors of less than one mark and two marks in cases (a) and (b), respectfully, to NMT 5% weighing error.

When a balance has been observed or suspected to have been moved, unleveled, contaminated, or otherwise damaged, misaligned, or misused, then its sensitivity must be tested and confirmed to be accurate.

Arm ratio test: The arm ratio test will determine whether both arms of the balance are of equal length.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the marker plate.
3. Place a 30-g weight in the center of each pan.
4. When the balance comes to rest, record the rest point.
5. If the rest point has changed from the middle of the marker plate, place a 30-mg weight on the lighter side.
6. When the balance comes to rest, this new rest point should either return to or go farther than the middle of the marker plate.

Shift test: The shift test checks the mechanics of the arm and lever components of the balance.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the marker plate.
3. Place a 10-g weight in the center of the left pan and place another 10-g weight successively toward the right, left, front, and back side of the right pan, noting the rest point in each case.
4. In any case where the rest point has changed from the center of the marker plate, add a 10-mg weight to the lighter side.
5. When the balance comes to rest, this new rest point either should return to or go farther than the middle of the marker plate.
6. Level the balance and adjust the balance until the pointer is in the middle of the marker plate.
7. Repeat the procedure with the 10-g weight in the center of the right pan, and vary the position of the 10-g weight on the left pan.
8. Level the balance and adjust the balance until the pointer is in the middle of the marker plate. Make several observations in which both 10-g weights are shifted simultaneously to off-center positions on their pans (i.e., both toward the inside, both toward the outside, one front and the other back). In any case where the rest point is shifted from the middle of the marker plate, the addition of a 10-mg weight on the lighter side should equalize or overcome the shift.

Rider and graduated beam test: The rider and graduated beam test checks the accuracy of the calibrated dial or rider on the balance.

1. Level the balance.
2. With the pans empty, adjust the balance until the pointer is in the middle of the index plate.
3. Place a 500-mg weight on the left pan and move the dial or rider to the 500-mg point.
4. When the balance comes to rest, record the rest point.
5. If the rest point has changed from the middle of the index plate, place a 10-mg weight on the lighter side.
6. When the balance comes to rest, this new rest point should either return to or go farther than the middle of the index plate.
7. Follow the same procedure using a 1-g weight on the left pan and the dial or rider on the 1-g point. If the new rest point is shifted from the middle of the index plate, a 10-mg weight to the lighter side should equalize or overcome the shift.

1.2 Electronic Balances

A typical electronic prescription balance is an instrument that provides essential readability for materials weighed within the range of capacities for the balance. The display should have prompts to guide users through the balance function, as well as an output port for printing if necessary. Most balances sold for prescription compounding meet or exceed Class I or II accuracy requirements according to NIST Handbook 44 and come with certificates issued under the National Type Evaluation Program (NTEP) of the National Conference on Weights and Measures.

Calibration/certification of the balance should be performed according to the standard operating procedures of the facility. Many electronic balances contain internal calibration programs that automatically calibrate the balance daily. If there is no internal calibration feature, external calibration may be conducted using a calibration weight, according to the procedure supplied by the manufacturer. Weights for use in calibrating an electronic balance should be kept in a special rigid and compartmentalized box and handled with plastic or plastic-tipped forceps, or gloves that are provided with the weights, to prevent scratching or soiling. These calibration weights should meet or exceed ASTM Class 1 criteria. In addition, there are companies

that offer calibration services to certify that the balance is performing adequately. For more information regarding the use of electronic balances, see ⟨41⟩ and *Weighing on an Analytical Balance* ⟨1251⟩.

1.3 Minimum Accurately Weighable Quantity on the Balance

The minimum accurately weighable quantity (MAWQ) is the smallest weight or mass that will produce no greater than a predetermined fraction of error on a properly calibrated, situated, and operated balance. The predetermined weighing error is assigned based on either a professional standard (such as NMT 0.05 or 5% error in the weight of any prescription ingredient) or scientific rigor, for example, NMT 0.005 or 0.5% error in the weight of an ingredient that is in limited supply. The compounder should use professional judgment when assigning the acceptable error for each process.

The formula for determining MAWQ for a typical Class III torsion balance is:

MAWQ = Sensitivity requirement/Acceptable error

Example: Calculate the MAWQ for a Class III torsion balance with a sensitivity requirement of 6 mg and an acceptable error of 5% or 0.05.

MAWQ = 6 mg/0.05 = 120 mg

For electronic balances, the MAWQ is calculated using the linearity or the absolute error over the range of the balance. This value is provided by the balance manufacturer. Note that the balance linearity and the readability of the smallest mass unit may not be the same.

Example: Calculate the MAWQ for an electronic balance with a linearity of 0.002 g and an acceptable error of 5% or 0.05.

MAWQ = 0.002 g/0.05 = 0.04 g or 40 mg

2. VOLUMETRIC APPARATUS

An assortment of appropriate volumetric devices should be available to accurately measure fluids and liquids of different volumes and densities. Pharmaceutical devices approved for measuring volumes of liquids, including burets, pipets, and cylindrical graduates marked in metric or metric and apothecary units, are to meet the standard specifications for glass volumetric apparatus described in "Specifications and Tolerances for Reference Standards and Field Standard Weights and Measures, 2. Specifications and Tolerances for Field Standard Measuring Flasks".[2] Conical graduates are to meet the standard specifications described in NIST Handbook 44.[1] There are ASTM standards (ASTM E542) for the calibration of laboratory volumetric apparatus that may be useful to compounders as well.[3]

2.1 Selection and Use of Graduates

CAPACITY

The capacity of a cylindrical graduate is the volume at the maximum graduation mark at the specified temperature. Volumes for prescription compounding and dispensing that are measured in cylindrical graduates should be adequate to not exceed 5% error.

CYLINDRICAL AND CONICAL GRADUATES

The error in a measured volume caused by a deviation of ±1 mm in reading the lower meniscus in a graduated cylinder remains constant along the height of the uniform column. The same deviation of ±1 mm causes a progressively larger error in a conical graduate, the extent of the error being further dependent upon the angle of the flared sides to the perpendicular of the upright graduate. A deviation of ±1 mm in the meniscus reading causes an error of approximately 0.5 mL in the measured volume at any mark on the uniform 100-mL cylinder graduate. The same deviation of ±1 mm can cause an error of 1.8 mL at the 100-mL mark on an acceptable conical graduate marked for 125 mL.

A general rule for selection of a graduate for use is to use the graduate with a capacity equal to or just exceeding the volume to be measured. Measurement of small volumes in large graduates tends to increase errors, because the larger diameter increases the volume error in a deviation of ±1 mm from the mark. The relation of the volume error to the internal diameters of graduated cylinders is based on the equation:

$$V = \pi r^2 h$$

V = volume

[2] Harris GL. Specifications and tolerances for reference standards and field standard weights and measures. 2. Specifications and tolerances for field standard measuring flasks. 1996. NTIS Order Number: PB96-178926. National Technical Information Service, Alexandria, Virginia 22312 (http://www.ntis.gov).

[3] ASTM E542-01. Standard practice for calibration of laboratory volumetric apparatus. West Conshohocken, PA: ASTM International; 2012. www.astm.org.

r = radius
h = height

An acceptable 10-mL cylinder having an internal diameter of 1.18 cm holds 109 µL in 1 mm of the column. Reading 4.5 mL in this graduate with a deviation of ±1 mm from the mark causes an error of about ±2.5%, and the same deviation in a volume of 2.2 mL in the same graduate causes an error of about ±5%. *Table 1* shows the accuracy limits for cylindrical graduates.

Table 1. Tolerance or Accuracy Limits for Class A Cylindrical Graduates[a]

Capacity (mL)	Smallest Graduation Mark Interval (mL)	Tolerance (±mL)[b]	Minimum Volume for 5% Error (mL)[c]
5	0.1	0.05	1.0
10	0.1 or 0.2	0.10	2.0
25	0.2 or 0.5	0.17	3.4
50	1	0.25	5.0
100	1	0.50	10.0
250	2	1.00	20.0
500	5	2.00	40.0
1000	10	3.00	60.0
2000	20	6.00	120.0
4000	50	14.50	290.0

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org. Some brands exceed the ASTM limits; for example, one source of a Class A 10-mL graduate lists the tolerance as ±0.08 mL.
[b] The constant volume error in each measurement.
[c] The minimum volume for N% error = [Tolerance (mL) × 100]/N (%); e.g., for N = 5% for a 10-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

There is an inverse relationship between the temperature and density of liquids. For compounding and dispensing purposes, deviations will be negligible when volume measurements are performed at temperatures NMT 5° from that specified on the particular cylindrical graduate, which is usually 20°. For example, the densities in g/mL of water, ethanol, and glycerin vary, respectively, from 0.999 to 0.997, 0.791 to 0.785, and 1.265 to 1.262 over the temperature range of 15°–25°. The accuracy of each cylindrical graduate used in pharmacy practice is recommended to be verified as follows, at a measuring temperature of 20°–25° with the assumption that deviations follow a normal or Gaussian distribution:

1. Tare a clean, dry graduate on a properly calibrated balance of adequate capacity, linearity, and readability for the volumes to be measured.
2. Fill the tared, dry graduate identically five or more times on a level surface to the smallest, a mid-range, and the maximum capacity graduation marks with *Purified Water* or deionized water, precluding and wiping spills and splashes from the exterior and interior above the target fill line.
3. Record the weight of each filling.
4. Calculate the mean weight of each set of fillings.
5. Calculate the percent deviation of each weight from the theoretical weight as follows:

   Percent deviation weight = {[Actual weight − (intended volume × 0.9975)]/(Intended volume × 0.9975)} × 100%
6. Calculate the percent mean deviation weight as follows:

   Percent mean deviation weight = {[Mean actual weight − (intended volume × 0.9975)]/(Intended volume × 0.9975)} × 100%

Deviations for cylindrical graduates used in compounding and dispensing should not exceed 5.0% for individual weights or 2.5% for mean weights of the corresponding volumes of *Purified Water*. *Table 1* and *Table 2* show that a wider range of volumes can be measured in Class A cylindrical graduates compared to Class B. According to ASTM standards, Class A cylindrical graduates must be marked with the letter "A" to designate compliance with applicable construction and accuracy requirements. Class B cylindrical graduates are the same basic design as Class A cylindrical graduates and are considered to be for general purpose use. However, volumetric tolerances may be up to twice the allowable range for Class A cylindrical graduates.

Table 2. Tolerance or Accuracy Limits for Class B Cylindrical Graduates[a]

Capacity (mL)	Smallest Graduation Mark Interval (mL)	Tolerance (±mL)[b]	Minimum Volume for 5% Error (mL)[c]
5	0.1	0.10	2.0
10	0.1 or 0.2	0.2	4.0

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org.
[b] The constant volume error in each measurement.
[c] The minimum volume for N% error = [Tolerance (mL) × 100]/N (%); e.g. for N = 5% for a 5-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

Table 2. Tolerance or Accuracy Limits for Class B Cylindrical Graduates[a] *(Continued)*

Capacity (mL)	Smallest Graduation Mark Interval (mL)	Tolerance (±mL)[b]	Minimum Volume for 5% Error (mL)[c]
25	0.2 or 0.5	0.34	6.8
50	1	0.50	10.0
100	1	1.00	20.0
250	2	2.00	40.0
500	5	4.00	80.0
1000	10	6.00	120.0
2000	20	12.00	240.0
4000	50	29.00	580.0

[a] Adapted from ASTM E1272-02. Standard specification for laboratory glass graduated cylinders. West Conshohocken, PA: ASTM International; 2012. www.astm.org.
[b] The constant volume error in each measurement.
[c] The minimum volume for N% error = [Tolerance (mL) × 100]/N (%); e.g. for N = 5% for a 5-mL graduate, the minimum volume is 2.0 mL. [The minimum volume for 5% error = (0.1 mL × 100)/5 = 2.0.]

2.2 Medicine Droppers

Medicine droppers meet the specifications in *Packaging and Storage Requirements* ⟨659⟩. Medicine droppers should be used only for qualitative purposes, such as pH adjustment with an acid, alkali, or buffer, and visual identification testing with reagents. Medicine droppers are not approved for volumetric measurements for compounding. Calibrated medicine droppers have markings to guide delivery of the prescribed quantity of medication. Their purpose should be limited to the measurement of a dose for administration.

2.3 Dispensing Bottles

Some dispensing bottles may be supplied by the manufacturer as part of the packaging, whereas others may be selected and supplied by the pharmacist. These containers are not necessarily accurately calibrated and should not be used for measurement during compounding and dispensing unless they are appropriately calibrated by the compounder.

2.4 Syringes

Syringes are available in a variety of sizes with calibrated increments used for measuring and may be used to accurately measure and deliver a wide range of liquid volumes. For viscous liquids, measurements made with syringes are usually more accurate than those made with cylindrical graduates.

ORAL SYRINGES

Oral syringes are available as a device for accurately providing a dose of liquid medication to a patient. The performance of the syringes may be user dependent. For this reason, it is important that suitable operating procedures are documented and followed, and that operators are specifically trained in the correct use of the instruments. Users should be cautious about relying on manufacturers' performance figures. It is more appropriate to perform calibration, taking into account the variation between different users. Oral syringes provide an improvement in dosing accuracy for viscous medications, compared with medication droppers.

SYRINGE CALIBRATION

Syringe calibration is based on the gravimetric determination of the quantity of water either contained or delivered, and the conversion of this value to true volume at the standard temperature of 20°. At 80% of the nominal syringe volume, 5–10 measurements should be performed.

Calibration procedure:

1. Aspirate and dispense an exact volume of deionized water that is 80% of the nominal syringe volume.
2. Determine the mass of the dispensed water.
3. Calculate the volume of the dispensed water using mass and density.
4. Document the measurement values.
5. Calculate the accuracy (R).

Calibration formula:

General Chapters

Accuracy (R) = [(Average value − target value)/Target value] × 100

2.5 Pipets

Pipets are thin glass tubes used to deliver volumes <25 mL. The two types of pipets are the single-volume pipet and the calibrated pipet. The single-volume pipet is the most accurate and the simplest to use, but the single-volume pipet is limited to the measurement of a single fixed volume; it is not capable of partial volume measurements.

The calibrated pipet has graduation marks from a point near the tip of the pipet to the capacity of the pipet. In addition to delivering its entire contents, the calibrated pipet can be used to deliver partial volumes with good volumetric precision.

Micropipets generally are used when very small volumes (<1 mL) are required; micropipets are available in a variety of sizes. Each micropipet can be adjusted, usually by turning a dial, to deliver a volume within a limited range. For example, one micropipet may deliver volumes of 0–20 µL, another delivers 0–100 µL, and yet another delivers 0–1000 µL. The pipet selected should provide the greatest accuracy for the volume to be measured. Pipets should be calibrated/certified periodically as specified in the facility's standard operating procedures. Calibrating micropipets is a very specialized process that requires adequate training and appropriate equipment. There are companies that offer contract calibration services to certify that pipets are performing correctly.

2.6 Volumetric Flasks

Volumetric flasks have a slender neck and wide, bulb-like base. They are single-volume glassware and come in a variety of sizes. Only one calibration mark is etched on the neck of the flask. When the flask is filled to that mark, the flask contains the volume indicated on the flask. Volumetric flasks are difficult to use if dissolving solids in liquid because of the narrowness of the neck. If solids are to be dissolved, the flask should be partially filled with liquid, the solid material added and completely dissolved, and then the flask should be filled to the calibration mark.

2.7 Additional Considerations When Using Volumetric Apparatus

The use of volumetric apparatus requires some working knowledge of viscosity, density, surface tension, and adhesion. Each of these properties may affect measurement accuracy. For example, higher-viscosity liquids will be drawn into the vessel at a slower rate, and the operator should allow time for complete filling. Delivery can also be slow, because higher-viscosity fluids travel through the orifice at a slower rate. Density can affect filling and emptying in a manner similar to viscosity. In addition, liquids with very low surface tension may tend to "crawl up" the vessel wall or leak from the tip of a syringe or pipet. Adhesion can affect accuracy by resulting in a slow rate of vessel emptying. The operator should confirm that all of the liquid has been drained from the vessel if performing a quantitative transfer.

Understanding the terms "To Contain (TC)" and "To Deliver (TD)" is important, because they apply to glassware. TC and TD glassware consists of vessels that range from 1 to about 100 mL and are individually marked to indicate whether they are TC or TD vessels. A TC vessel is designed to deliver the entire measured content of the operation, and it may require forced air to expel the final quantity. A TD vessel is designed to deliver the entire measured amount via gravity flow.

GLOSSARY

Accuracy: The closeness of the displayed weight, as measured by the balance, to the true weight, as known by the use of a calibration weight or weights.

Balance indicator: A combination of elements, one or both of which will oscillate with respect to the other, to indicate the equilibrium state of the balance during weighing.

Capacity: Maximum weight, including the weight of tares to be placed on one pan. The nominal capacity of a prescription scale (Class III prescription balance) is assumed to be one-half apothecary ounce (15.55 g), unless otherwise marked. [NOTE—This is applicable only to scales not marked with an accuracy class. Past and currently available Class III prescription balances typically have a capacity of either 60 or 120 g and bear a label stating such.]

Linearity: The ability to maintain the same sensitivity over the entire weighing range of the balance. This is the constant weighing error in every amount weighed within the capacity range of the balance. This term is used only with electronic balances. Analogous terms are "absolute error" or "linear accuracy". The linearity is determined by the balance manufacturer.

Minimum accurately weighable quantity (MAWQ): The smallest weight or mass that will produce no greater than a predetermined fraction of error on a properly calibrated, situated, and operated balance.

Precision: The reproducibility of the weighing measurement as expressed by a standard deviation. A similar term, "repeatability", is sometimes used in specifications for electronic balances.

Readability: The smallest division at which the balance increments.

Repeatability: An instrument's ability to consistently deliver the same weight reading for a given object, and to return to a zero reading after each weighing cycle. This is tested by repeatedly weighing the same test weight.

Rest point: The point on the index plate at which the indicator or pointer stops when the oscillations of the balance cease, or the index plate position of the indicator or pointer calculated from recorded consecutive oscillations in both directions past the zero of the index plate scale. If the balance has a two-pointer indicating mechanism, the position or the oscillations of only one of the pointers need to be recorded or used to determine the rest point.
Sensitivity requirements (SR): The maximum change in load that will cause a specified change, such as a particular quantity of pointer scale division marks, of the pointer or indicating component of the balance.
Tare bar: An auxiliary ungraduated weighbeam bar with a movable poise. It can be used to correct for variations in weighing glasses or papers.
Weighbeam or Beam: A graduated bar equipped with a movable poise or rider. Metric graduations are in 0.01-g increments up to a maximum of 1.0 g.

〈1177〉 GOOD PACKAGING PRACTICES

This chapter provides general guidance on packaging considerations for Pharmacopeial preparations that may be stored, transported, and distributed. It describes procedures that should be considered to ensure that proper packaging practices are maintained. It does not affect any applicable requirements under good manufacturing practices, state laws governing pharmacy, the *USP General Notices and Requirements* or monographs, or provisions under approved labeling.

Definitions for storage conditions and packaging are provided in 〈659〉 *Packaging and Storage Requirements*. All equipment used for recording, monitoring, and maintaining these temperature and humidity conditions should be calibrated on a regular basis. This calibration should be traceable to national or international standards (see also the general information chapter *Monitoring Devices—Time, Temperature, and Humidity* 〈1118〉).

CONTAINERS

The monograph packaging and storage statement specifies that the container (primary package) should meet the requirements under *Containers—Glass* 〈660〉, *Plastic Packaging Systems and their Materials of Construction* 〈661〉, *Plastic Materials of Construction* 〈661.1〉, *Plastic Packaging Systems for Pharmaceutical Use* 〈661.2〉, and *Containers—Performance Testing* 〈671〉, which include the stipulations for determining if a container is "tight" or "well-closed." In most cases, compendial preparations are expected to be packaged in "tight" containers, especially if the article is moisture sensitive. In addition, where necessary, the packaging component should protect the preparation from light, reactive gases, solvent loss, microbial contamination, etc. "Tight" and "well-closed" containers are clearly defined in 〈659〉 *Packaging and Storage Requirements*, whereas testing protocol and moisture permeation limits to determine if the container meets either of these definitions can be found in *Containers—Glass* 〈660〉, *Plastic Packaging Systems and their Materials of Construction* 〈661〉, *Plastic Materials of Construction* 〈661.1〉, *Plastic Packaging Systems for Pharmaceutical Use* 〈661.2〉, and *Containers—Performance Testing* 〈671〉 for single-unit and multiple-unit containers.

A packaging system is composed of a container system with its closure. This system may include several layers of protection for the Pharmacopeial preparation along with any sealing devices, delivery devices, labeling, and package inserts. The *General Notices* section also provides definitions for types of packaging systems that contain and protect a Pharmacopeial preparation (e.g., single-unit containers, unit-dose containers, etc.). Stability testing is conducted on the dosage forms packaged in the container–closure system proposed for marketing.

One type of permeation test for multiple-unit containers is described in *Containers—Performance Testing* 〈671〉. This test is intended for drug products being dispensed on prescription in vials with a container–closure system. The results of the test reflect the water vapor permeation through the container and through the closure. Limits have been established to define whether a container for dispensing has tight or well-closed characteristics with regard to water vapor permeation. FDA recommends that manufacturers perform this test on the container–closure system, although it is not specified in *USP*. In this particular test, the inner seal of the manufacturer's container–closure system is removed prior to testing.

Single-unit containers for capsules and tablets under *Containers—Performance Testing* 〈671〉 are measured for water vapor permeation according to the criteria for the four classes of containers (classes A–D).

The *USP* recognizes several official container materials that can be selected on the basis of their properties. Most containers are made of glass or plastic. Glass containers must be evaluated for chemical resistance and light transmission (if indicated) as described in *Containers—Glass* 〈660〉. In addition, injectable medication containers should be reviewed according to the section *Packaging and Storage Requirements* 〈659〉, *Injection Packaging*. Elastomeric closures should be evaluated separately as described in *Elastomeric Closures for Injections* 〈381〉. Plastic containers should be assessed as described in *Plastic Packaging Systems and their Materials of Construction* 〈661〉; *Plastic Materials of Construction* 〈661.1〉; and *Plastic Packaging Systems for Pharmaceutical Use* 〈661.2〉. As articulated in these sections, plastics should undergo testing for light transmission (if appropriate), water vapor permeation (see also *Containers—Performance Testing* 〈671〉), extraction physiochemical testing, and biological testing (see also *Biological Reactivity Tests, In Vitro* 〈87〉 and *Biological Reactivity Tests, In Vivo* 〈88〉). For example, testing water vapor

General Chapters

permeation for a PE container is conducted by sealing the container with heat-sealed foil laminate and measuring the water permeation in a humid atmosphere. Given that water vapor does not permeate the foil laminate, this test assesses only the properties of the container. The level of protection provided by a packaging system marketed with a heat-sealed foil laminate inner seal (prior to removal of the foil) is approximated by this test. However, in the case of a PET bottle for liquid preparations, water vapor permeation testing is done by filling containers with water and measuring the water loss rate in a dry atmosphere. Additional testing may be required for certain pharmaceutical dosage forms as well.

The container–closure system for the storage or shipment of a bulk liquid drug substance is typically plastic, stainless steel, a glass-lined metal container, or an epoxy-lined metal container with a rugged, tamper-resistant closure. Qualification of the container–closure system for these types of preparations includes evaluation for solvent and gas permeation, light transmittance, closure integrity, ruggedness in shipment, protection against microbial contamination through the closure, and compatibility and safety of the packaging components as appropriate (see *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩).

Other information on container–closure systems may be found in FDA's *Guidance for Industry: Container Closure System for Packaging Human Drugs and Biologics,* www.fda.gov.

PACKAGING

Packaging for Pharmacopeial articles can be divided into categories according to terminology generally accepted by industry. As mentioned earlier, the *General Notices* section provides some definitions for different types of containers classified by their characteristics and uses. In addition, the ASTM Committee D10 on packaging publishes terminology, practices, test methods, specifications, guides, and classifications for testing and evaluating packaging (see ASTM D99695, "Standard Terminology of Packaging and Distribution Environments"). Under certain rules and guidelines (e.g., such as 49 CFR, Dangerous Goods, and others), however, alternate terminology is used for the components described below. For terminology pertaining to repackaging processes, refer to *Packaging and Repackaging—Single-Unit Containers* ⟨1136⟩.

Primary Container

This container is in direct contact with the Pharmacopeial preparation. The purpose of a primary container, also referred to as an immediate container, is to protect the preparation from environmental hazards during storage and handling. In some cases, the primary container is also a specialized delivery system, such as an aerosol or a metered-dose dispenser (see *Pharmaceutical Dosage Forms* ⟨1151⟩). For the majority of oral dosage forms, the primary container consists of a cap and a bottle or a blister or pouch package that can be made from many different materials, including glass, plastic, single or laminated flexible materials, and metal. All components of the primary container must meet the requirements under 21 CFR for direct food contact and, where applicable, the *USP* requirements under *Containers—Glass* ⟨660⟩, *Plastic Packaging Systems and their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩, and *Containers—Performance Testing* ⟨671⟩. A full description of the primary container is included under the "Container/Closure System" section of the New Drug Application (NDA), Abbreviated New Drug Application (ANDA), or other classes of FDA submissions.

Critical Secondary Container

This container is not in direct contact with the article, but it provides essential product stability protection. For example, a primary container may be packed inside a critical secondary container such as a pouch to provide moisture, gas, light, or microbial protection not afforded by the primary container. A description of the critical secondary container is included under the "Container/Closure System" section of the NDA, ANDA, or other classes of FDA submissions.

Secondary Container

This container encloses one or more primary containers. A secondary container is not always present. If used, it is usually designed for the final market presentation. Secondary containers are often used simply to carry required labeling or to keep individual primary containers together with delivery systems or other add-on features. Secondary containers can also provide protection against damage in the handling and distribution system. The most common secondary container is the standard folding carton. Some products, such as syringes, may be placed in trays prior to packing in a carton. Secondary container materials are not included in the container and closure description and require neither stability studies nor prior approval when making a change in the materials used.

Additional Packaging

A wide variety of additional packaging, such as trays and display cartons, may be used to hold primary containers.

Unit of Sale

This may be an individual bottle, a carton containing one or more bottles, or a tray with multiple primary containers. A unit of sale may contain more than one item for individual sale. For example, a display tray may have multiples of a single article or a variety of related articles from a single manufacturer, each intended for individual sale. The individual item intended for sale is referred to as a stock-keeping-unit (SKU). SKUs are distinguished by a discrete National Drug Code (NDC). Over-the-counter (OTC) articles contain a Universal Product Code (UPC) for all SKUs. A prescription SKU may be intended for final consumer use and may not be repackaged by a pharmacy. Such packages, often called "unit of issue" or "unit of use," require child-resistant (CR) packaging as described under 16 CFR 1700, "Poison Prevention Packaging," except for packages exempted by the Consumer Product Safety Commission. The CR feature is typically incorporated by the manufacturer (see *Packaging and Repackaging—Single-Unit Containers* 〈1136〉). OTC articles are regulated under the same rule, but only if they contain certain active ingredients above specified limits. Any regulated product shipped via the United States Postal Service (USPS) must meet the USPS rules under 39 CFR 111.

Final Exterior Package

This is typically a corrugated fiberboard box (case) or a wrapper. The shipping case label is affixed to this outermost layer and incorporates all of the bar codes required by the National Wholesale Druggists' Association (NWDA). This final package is normally shipped on pallets to distribution centers, wholesalers, and other large-volume customers. The manufacturer may or may not intend that this package enter the small-package-shipping environment as an individual unit without further protection.

Especially with fiberboard boxes, relative humidity (RH) may have a negative effect on the compression strength of the box, causing loads to shift and potentially damage the article or the outer and inner packaging. Articles stored in refrigerators or freezers, which are environments with high RH, are prone to this type of damage when stacked. The problem may be exacerbated by carton design, stacking pattern, or use of low edge-crush-test corrugated fiberboard. Computer programs are available to determine the acceptable stack height and patterns on the basis of carton weight, style, size, and material. If problems occur, the product manufacturer should be contacted. Source materials and reference information on corrugated fiberboard boxes can be found in the "Fiber Box Handbook" published by the Fiber Box Association.

A wholesaler or other reshipper should not assume that the package received from the manufacturer is suitable for reuse. Many packages are customized for very specific routes and modes of transportation and are not suitable for other applications. Like any other shipping container, insulated cartons and inner protective packaging can be damaged during transit, thus affecting package performance and possibly allowing damage to contents if reused.

ENVIRONMENTAL ISSUES

Packaging materials are regulated by a variety of federal, state, and local rules. In general, most pharmaceutical packaging containers can be recycled within local programs. The use of recycled material in primary containers is governed by the FDA, but it is generally not allowed. Pharmaceutical manufacturers commonly follow the most current Coalition of Northeastern Governors' rules (e.g., Model Toxics in Packaging Legislation) regarding heavy metals in packaging and other environmental issues.

Certain classes of Pharmacopeial articles may require special handling. Such articles include products classified as (1) Dangerous Goods under the Department of Transportation (DOT), state, local, or carrier rules; (2) controlled drugs under the Drug Enforcement Administration (DEA); or (3) scheduled substances under state regulations.

LABELING

The labeling of shipping containers by manufacturers must be in compliance with the pertinent sections of FDA and DOT rules.

Dangerous Goods

The labeling of shipments classified as *Dangerous Goods,* including all information on the bill of lading or airway bill, must follow the instructions provided by the DOT, the International Air Transport Association (IATA), and the carrier. The exterior package must carry all of the required standard symbols for the class of goods, and the shipping container must comply with the performance standards for the articles enclosed. The shipper of record is responsible for compliance with the Dangerous Goods requirements.

Controlled Substances

When Pharmacopeial preparations that contain DEA-scheduled controlled substances are distributed to a patient directly via the USPS, these articles must be marked and labeled in accordance with USPS Domestic Mail Manual, Regulation Article C023, Section 7.2.

⟨1178⟩ GOOD REPACKAGING PRACTICES

INTRODUCTION

This chapter is intended to provide guidance to those engaged in repackaging of oral solid drug products; and the chapter provides information to any person who removes drugs from their original container–closure system (new primary package) and repackages them into a different container–closure system for sale and/or for distribution.

This chapter does not apply to pharmacists engaged in dispensing prescription drugs in accordance with state practice of pharmacy. The pharmacist needs to apply

(1) the principal information provided in the USP general information chapters *Plastic Packaging Systems and Their Materials of Construction* ⟨661⟩, *Plastic Materials of Construction* ⟨661.1⟩, and *Plastic Packaging Systems for Pharmaceutical Use* ⟨661.2⟩ and

(2) other beyond-use date references in the subsection *Expiration Date and Beyond-Use Date* in the *Labeling* section under *General Notices and Requirements*.

ESTABLISHING EXPIRATION DATE

In the absence of stability data, the following criteria should be considered by repackagers when assigning an expiration date.

Unit-Dose Packaging

1. The original container–closure system of the drug product to be used for repackaging must be received un-opened and show no outward signs of having been previously opened.
2. The unit-dose container–closure system must meet the testing requirements under *Containers—Performance Testing* ⟨671⟩ for either *Class A* or *Class B* containers.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The unit-dose container–closure system must meet or exceed the original container's specification for light resistance.
5. The conditions of storage must meet the storage specifications provided in the *USP General Notices* and as described in the labeling of the original container–closure system received for repackaging. Where no specific storage conditions are specified, the product must be maintained at controlled room temperature and in a dry place during the repackaging process, including storage.
6. The expiration dating period used for the repackaged product does not exceed (1) 6 months from the date of repackaging; or (2) the manufacturer's expiration date; or (3) 25% of the time between the date of repackaging and the expiration date shown on the manufacturer's bulk article container of the drug being repackaged, whichever is earlier.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

Multiple-Unit Packaging

1. A repackager may use the manufacturer's original expiration date without additional stability testing if the drug product is repackaged into an equivalent container–closure system that is at least as protective as, or more protective than, the original system and complies with criteria established for equivalency.

2. The original container–closure system of the drug product to be used for repackaging must be received un-opened and shows no outward signs of having been previously opened.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The conditions of storage meet the storage specifications in the *USP General Notices* and as described in the labeling of the original container–closure system received for repackaging. When no specific storage conditions are specified, the product should be maintained at controlled room temperature and in a dry place during repackaging operations.
5. The type of container–closure system used for repackaging must be at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system.
6. The container–closure system must meet or exceed the original container–closure system's results for light transmission.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

REFERENCES FOR REPACKAGING REGULATIONS AND GUIDANCES

The references listed below are not meant to be all inclusive: specific repackaging operations may have additional requirements.

- **Food, Drug, and Cosmetic Act**
- **Food and Drug Administration Regulations and Guidances**
  Enforcement Policy: 21 CFR, Part 7
  General Labeling Provisions: 21 CFR, Part 201, Subpart A
  Drug Establishment Registration and Listing: 21 CFR, Part 207.20
  Current Good Manufacturing Regulations: 21 CFR, Parts 210–211
  Special Requirements for Specific Human Drugs: 21 CFR, Part 250
  Controlled Substances: 21 CFR, Part 1300
  Potable Water: 40 CFR, Part 141
  FDA *Compliance Policy Guides*, including the following:
    Sub Chapter 430 Labeling and Repackaging
    Sub Chapter 460 Pharmacy Issues
    Sub Chapter 480 Stability/Expiration Dating
- **Applicable USP Chapters**
  ⟨660⟩ *Containers—Glass*
  ⟨661⟩ *Plastic Packaging Systems and Their Materials of Construction*
  ⟨661.1⟩ *Plastic Materials of Construction*
  ⟨661.2⟩ *Plastic Packaging Systems for Pharmaceutical Use*
  ⟨671⟩ *Containers—Performance Testing*
  ⟨1079⟩ *Good Storage and Distribution Practices for Drug Products*
  ⟨1136⟩ *Packaging and Repackaging—Single-Unit Containers*

GLOSSARY

⟨659⟩ *Packaging and Storage Requirements* provides definitions related to repackaging. For the purposes of this chapter, a repackager, a contract packager, and an equivalent container–closure system are defined as follows:

Repackager: A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient-specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice Regulations in 21 CFR 210 and 211.

Contract packager: A contract packager is an establishment that is contracted to package or repackage a drug product into a single- or multi-unit container. These containers should meet all of the applicable requirements in this chapter. A contract packager does not take ownership from the manufacturer and generally receives the assigned expiration date from the contractor.

General Chapters

Equivalent container–closure system: This term refers to a container–closure system that is at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system. These values may be determined by the repackager, or they may be obtained from the container–closure vendor for the specific container–closure system under consideration.

⟨1180⟩ HUMAN PLASMA

SCOPE

This chapter provides a consolidated source of information regarding human plasma, with emphasis on plasma for fractionation. Specifically, the chapter addresses plasma classification and nomenclature; collection and processing procedures required for ensuring product safety; details of specific plasmas; and quality systems relating to plasma collection. The chapter also includes, at the end of the text sections, a glossary; a list of abbreviations used in the chapter; and appendices that provide plasma definitions, donor selection criteria, and testing requirements.

Plasma originating from U.S.-licensed collection facilities provides the major supply for the global plasma derivative market. The U.S. Food and Drug Administration (FDA) regulates the collection and processing of plasma used for further manufacture. Title 21 of the Code of Federal Regulations (CFR) details Good Manufacturing Practice (GMP) requirements and product standards to protect the health of the blood donor and to ensure the safety and efficacy of blood products. CFR regulations are updated periodically, but between revisions, existing regulations may not address the most current issues and scientific developments. Therefore, the FDA periodically publishes guidance documents.[1] (For further information, see *Quality Systems*, below).

This chapter emphasizes U.S. practices, but because plasma and its derivatives are shipped globally, it is important to recognize that there are regional differences in recommendations, requirements, and regulations. Therefore this chapter provides information regarding the European Union (EU), the United Kingdom (UK), and Australia.

OVERVIEW

Composition of Plasma

Plasma constitutes approximately 55% of the total blood volume. It is a clear, straw-colored, complex liquid that is 7% protein, 91% water, and 0.9% mineral salts. The majority (approximately 70%) of total plasma protein is albumin. Additional plasma proteins relevant to fractionation include immunoglobulins, coagulation factors, fibrinolytic proteins, proteases, and protease inhibitors. These constituent plasma proteins can be isolated on the basis of the different solubility characteristics of each protein when subjected to specific conditions of pH, temperature, ionic strength, and ethanol concentration. The major products derived from fractionation are listed in *Table 1*.

Table 1. The Major Fractions and Products from the Cohn Process

Fraction	Product
Cryoprecipitate	Antihemophilic factor (FVIII)
Cryosupernatant	Antithrombin III, factor IX complex
Fraction I	Fibrinogen, factor XIII
Fraction II	Immune globulin G (IgG)
Fraction III	IgA, IgM, prothrombin, plasminogen
Fraction IV-1	Factor IX complex, activated factor IX complex
Fraction IV-4	Plasma protein fraction, alpha-1 proteinase inhibitor
Fraction V	Albumin

Plasma for Manufacture of Derivative Products

The two methods for collection of human plasma are automated apheresis (for definitions, see the *Glossary* preceding the appendices) and centrifugation of whole blood donations. Source Plasma collected by apheresis constitutes the majority of plasma used in the manufacture of plasma derivatives in the United States. Plasma collected for transfusion but not so used (i.e., recovered plasma) also may be used for manufacture. Flow charts delineating how apheresis and whole blood–derived

[1] FDA. Blood Guidances. www.fda.gov/cber/blood/bldguid.htm. FDA Memoranda to Blood Establishments available at www.fda.gov/cber/memo.htm.

plasma can be used in the manufacture of plasma derivatives in the United States and Europe are presented in *Figures 1* and *2*, respectively.

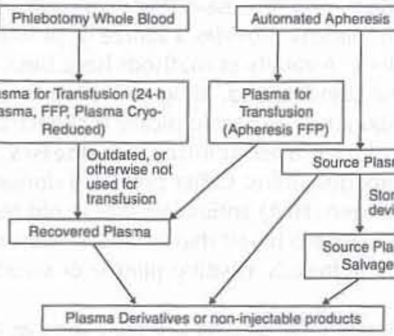


Figure 1. U.S. plasma derivative manufacture: FDA standards.

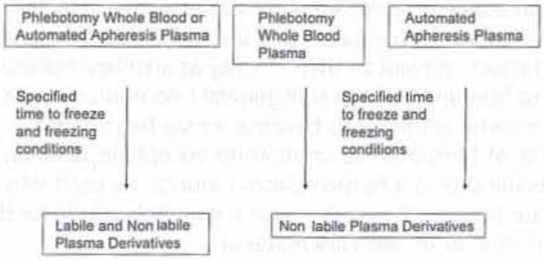


Figure 2. EU plasma derivative manufacture: EU standards.

Regardless of the collection method, plasma for fractionation should be a clear to slightly turbid liquid without visible sign of hemolysis; it may vary in color from light yellow to green; it should be ±10% of the stated volume; and it should show no sign of clots.

Source Plasma

Licensed Source Plasma may be manufactured only in collection centers that are approved by the FDA for the collection and distribution of Source Plasma in interstate commerce. Currently, federal regulations governing the manufacture of Source Plasma, including minimal requirements for donors, are found in 21 CFR 640, Subpart G. By definition, Source Plasma is plasma intended for further manufacture. Source Plasma donors can donate as often as twice a week and may be compensated. In addition to FDA requirements, most plasma collectors and fractionators also comply with voluntary standards established by the Plasma Protein Therapeutics Association (PPTA), a trade and standards-setting organization.[2] PPTA voluntary standards address several areas of donor, plasma unit, plasma pool, and center management, and are designed to supplement existing regulatory requirements.

Plasma for Transfusion

Plasma for transfusion is not intended for further manufacture but for direct transfusion to patients. It may be collected by either whole blood or apheresis donation. In the United States, plasma for transfusion comes from unpaid volunteer donors. Blood collecting facilities that collect plasma for transfusion typically comply with requirements of both the CFR and a voluntary trade organization, the American Association of Blood Banks (AABB). Currently, sections 640.3 and 640.31 of 21 CFR outline requirements for donors of whole blood and therefore govern most donors of plasma for transfusion, regardless of the collection method. AABB voluntary standards include information contained in FDA regulations and guidance pertaining to blood and plasma, as well as additional standards.[3] Plasma for transfusion may be converted to recovered plasma, an unlicensed product that may be used for further manufacture.

Plasma for Ancillary Use in Biologics Manufacturing

Human plasma and its derivatives are used in the manufacture of other biologic products. In this role, the plasma or plasma derivative falls into the category of ancillary use. This is defined as use of a reagent or material as a processing or purification

[2] PPTA. www.pptaglobal.org.
[3] AABB. *Standards for Blood Banks and Transfusion Services.* 25th ed. Bethesda, MD: AABB; 2008.

aid or a reagent that exerts an effect on the therapeutic substance but is not intended to be part of the final product formulation (see the *USP* general information chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* ⟨1043⟩).

Human plasma is commonly used in manufacturing processes that involve primary human cells or cell lines intended for therapeutic applications. In these applications, plasma provides a source of protein and possibly other factors that enhance expansion and differentiation of cell populations. A variety of methods have been used to prepare human plasma and derivatives for ancillary use, but the practices are not standardized. Allogeneic plasma typically is obtained from either apheresis or whole blood, using citrate anticoagulation. Allogeneic plasma typically is collected from paid donors who, like Source Plasma donors, have been screened for the absence of transfusion-transmissible diseases. Preferred donors may be blood type group AB, because they lack anti-A and anti-B isohemagglutinins. Other preferred donors include untransfused males, because this group is unlikely to have human leukocyte antigen (HLA) antibodies that could react with cells in a given culture system. Serum is prepared either from nonanticoagulated whole blood that has been allowed to clot or by the addition of calcium to plasma obtained from citrated whole blood or apheresis. Heating plasma or serum to 56° inactivates heat-labile complement and other proteins.

Although there are no standardized specifications for plasma products used as ancillary materials, assays often include safety testing associated with general biologics (e.g., bacterial/fungal cultures, endotoxin, and mycoplasma). In addition, characterization of the products may include tests for irregular erythrocyte and HLA antibodies, osmolality, pH, total protein and immunoglobulin concentrations, hemoglobin concentration, and chemistries such as Na, K, Cl, Ca, and glucose.

In some instances, plasma from bovine sources has been used instead of human plasma. The differences between bovine and human plasma products include factors relevant to their efficacy as ancillary materials, as well as safety of the final manufactured product when administered to humans (see the *USP* general information chapter *Bovine Serum* ⟨1024⟩). Fetal bovine serum is preferred to human serum for some applications because it may be superior in promoting human cell growth in vitro. Safety considerations, especially the risk of transmissible spongiform encephalopathy and allergic reactions related to antibovine antibodies, have led to recommendations that human plasma sources be used whenever possible instead of bovine sources. However, substitution of human for bovine plasma does not completely eliminate the risk of infectious and immunologic sequelae, even when human plasma is used as an ancillary material.

PLASMA COLLECTION AND PROCESSING

This section discusses the standard principles involved in plasma collection and the methods used to ensure the safety of the plasma and subsequently manufactured plasma derivatives. Principles for screening and testing of donors are presented in other parts of this chapter.

Collection

Source Plasma is collected by apheresis. Recovered plasma can be obtained either by apheresis or as a by-product of whole blood collection. Collection should take place via an FDA-approved, closed, sterile, pyrogen-free collection system that contains an anticoagulant. No antibacterial or antifungal agent should be added to the plasma. Donations must be collected aseptically. The skin of the donor must be aseptically prepared. Source Plasma is collected using 4% sodium citrate as the anticoagulant. The composition of sodium citrate is given in *Table 2*.

Three anticoagulant solutions are licensed in the United States for collection of whole blood: citrate phosphate dextrose (CPD), citrate phosphate double dextrose (CP2D), and citrate phosphate dextrose adenine (CPDA-1). The composition of blood collection bags containing these anticoagulants is shown in *Table 3*. Plasma for transfusion or further manufacture can be made from a unit of whole blood collected in any of the three anticoagulant solutions. Regulations relating to plasma make no distinction among the three anticoagulant solutions. Consequently, collection, storage, and transport requirements are identical regardless of the anticoagulant solution used in the primary collection.

Table 2. Anticoagulant Solution for Collection of Source Plasma by Apheresis (4% Sodium Citrate)

Volume	Sodium Citrate Dihydrate	Citric Acid Anhydrous	pH (25°)	Ratio of Solution to Whole Blood
250 mL or 500 mL	40 g/L	As required for pH adjustment	6.4–7.5	1:16

Table 3. Anticoagulant Solutions Used during Whole Blood Collection for Recovery of Plasma (500-mL collection bags)

Anticoagulant	CPD[a]	CP2D[b]	CPDA-1[c]
Volume (mL)	70	63	63
Dextrose (mg)	1780	3220	2010
Sodium citrate dihydrate (mg)	1840	1660	1660

Note: Collection of Source Plasma typically involves the use of sodium citrate as the anticoagulant. The specification for sodium citrate is given in *Table 2*. Plasma for transfusion is stored at 2° to 8° after collection. Plasma collected by apheresis should be frozen immediately at −18° or colder.

[a] Citrate phosphate dextrose.

[b] Citrate phosphate double dextrose.

[c] Citrate phosphate dextrose adenine.

Table 3. Anticoagulant Solutions Used during Whole Blood Collection for Recovery of Plasma (500-mL collection bags) *(Continued)*

Anticoagulant	CPD[a]	CP2D[b]	CPDA-1[c]
Citric acid anhydrous (mg)	209	206	206
Monobasic sodium phosphate (mg)	155	140	140
Adenine (mg)	—	—	17.3
pH (25°)	5.3–5.9	5.3–5.9	5.3–5.9
Ratio of solution to whole blood	1.4:10	1.4:10	1.4:10

Note: Collection of Source Plasma typically involves the use of sodium citrate as the anticoagulant. The specification for sodium citrate is given in *Table 2*. Plasma for transfusion is stored at 2° to 8° after collection. Plasma collected by apheresis should be frozen immediately at −18° or colder.

[a] Citrate phosphate dextrose.

[b] Citrate phosphate double dextrose.

[c] Citrate phosphate dextrose adenine.

Labeling

The labeling for Source Plasma should comply with 21 CFR 640.70 and 21 CFR 640.69(b). The labeling for whole blood should comply with 21 CFR 606.121 and 606.122, and with internal licenses.

A unique identification number is assigned so that the donation can be related to the individual donor records and test results. The origin of each donation in a plasma pool and the results of the corresponding donation and laboratory tests must be traceable while the required degree of confidentiality concerning the donor's identity is maintained. Whole blood must be labeled "This Product may transmit infectious agents" [21 CFR 121(c)(9)]. Source Plasma or recovered plasma must be labeled "Caution: For Manufacturing Use Only" if the product is intended for use in fractionation. For plasma to be used as a reagent or for in vitro use, the required labeling statement is "Caution: For Use in Manufacturing Noninjectable Products Only" [21 CFR 121(e)(5)(ii)].

Storage

Plasma for fractionation should be stored at or below −20°. The plasma can still be used for fractionation if its temperature exceeds −20° on (at most) one occasion for not more than 72 hours and if the plasma has been maintained at a temperature of −5° or lower at all times. Storage temperatures must be maintained during transport.

PLASMA SAFETY CONSIDERATIONS

Plasma is protected by five overlapping safeguards that the FDA has termed the "Five-Layer Safety Net": donor screening, blood testing, donor deferral, quarantine, and investigation. For guidance in this area, see FDA Publication No. FS 02-1 February 2002.

Voluntary measures that provide an additional margin of safety include recruitment and retention of suitable donors and inventory hold procedures. In the manufacture of plasma-derived products, steps taken for viral clearance are very important for ensuring safety.

None of these measures is sufficient by itself; the safety net is the overlapping combination of the activities.

Donor Screening

The selection of a suitable site for blood and plasma donation activities is a first and very important step to ensure safe donations. Areas with low disease prevalence are preferred as locations for donation centers, thereby reducing the likelihood of collecting plasma from an infected donor.

One of the PPTA voluntary standards is the viral marker standard, which obliges plasma centers to report viral marker rates for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) in the donor populations. A center's rates are compared to the industry average. Alert limits are set to take into account the number of annual donations in the center. If a center exceeds the limit for any of these viruses or the aggregate of all three, the center must implement corrective actions that will bring the center into compliance with the standard.

Appropriate donor selection helps provide a safe plasma supply. A detailed donor history questionnaire in conjunction with a careful medical examination allows center personnel to recognize unsuitable donors whose behavior puts them at risk for transfusion-transmitted disease or who have underlying medical conditions that preclude donation.

An additional measure put in place by centers that collect Source Plasma is the PPTA National Donor Deferral Registry (NDDR). It lists donors throughout the United States who have been previously deferred from donation (although it provides no information about the reason for deferral). Other countries have different systems depending on their national regulations concerning personal data gathering. Any individual who tests positive for HIV, HBV, or HCV is entered into the national database (the National Donor Deferral Registry) used by all U.S. plasma centers that are certified under the International Quality Plasma Program (IQPP). All individuals who present at a U.S. plasma center for the first time are checked against the NDDR. In

this manner, donors who have previously been deferred for positive test results at any participating facility can be identified and rejected quickly. This standard ensures that donors deferred for positive test results do not donate in other facilities.

A voluntary safety initiative, the Qualified Donor Standard, implemented by the plasma fractionation industry, builds on the fact that many plasmapheresis donors contribute plasma frequently. A donor who enters a plasmapheresis center for the first time is called an Applicant Donor, and the first donation is used for further manufacturing only if the donor returns a second time. Potential donors must pass two separate medical screenings and testing for HIV, HBV, and HCV on two different occasions. Only after satisfactory screenings and negative test results does that person become a Qualified Donor. If a donor does not return within 6 months, that person loses his/her Qualified Donor status and must qualify again. This standard means that plasma from a one-time-only donor (even when all test results are negative) cannot be used for further manufacture. This standard results in committed donors and eliminates the risk that plasma centers will accept so-called test seekers. The interval between permitted donations of whole blood is too long to allow a similar screening program, although quite a number of donors in whole-blood donor centers are regular and repetitive donors.

Another PPTA voluntary standard addresses donor management criteria. The Community-Based Donor Standard allows only donors who permanently reside within its defined donor recruitment area to donate at a given center. In addition, a Donor Education Standard requires new donors to engage in an educational program and follow-up assessment regarding HIV/acquired immune deficiency syndrome (AIDS) and activities that place them at risk for HIV/AIDS.

In addition to donor management strategies and standards, PPTA has issued a plasma unit management standard called Inventory Hold. This standard states that collected plasma will be held in inventory for at least 60 days from the time of collection. This allows the retrieval of units as a result of post-donation information (information that was not known at the time of donation) that would have disqualified the donor. This information could include admitting high-risk behavior; becoming reactive for HIV, HBV, or HCV; or providing incorrect information about international travel.

Blood Testing

Testing of donations is an important safety measure both for plasma intended for transfusion and plasma intended for further manufacturing. Both enzyme-linked immunosorbent assays (ELISA) and nucleic acid amplification technologies (NAT) are used to screen donations for the presence of infectious disease. Automation provides the necessary throughput to screen every donation for a variety of potential pathogens.

Testing strategies differ depending on whether the plasma donation is intended for transfusion or further manufacture. Plasma for transfusion requires more extensive infectious disease testing, because there are no pathogen inactivation/removal technologies licensed for this product in the United States. On the other hand, plasma for further manufacture is subjected to several pathogen inactivation/removal steps during manufacture, thereby obviating the need for some disease testing. *Table 4* outlines current infectious disease tests required by the FDA for plasma donations collected in the United States. *Appendix 3* compares EU and U.S. disease testing and donor deferral requirements.

Table 4. FDA Disease Test Requirements for Plasma for Transfusion and Plasma for Further Manufacturing

Disease	Plasma for Transfusion	Plasma for Further Manufacturing[a]
Hepatitis B	Hepatitis B surface Antigen (HBsAg) Hepatitis B core antibody	HBsAg
Hepatitis C	Anti-HCV HCV RNA	Anti-HCV HCV RNA
HIV	Anti-HIV I/II HIV RNA	Anti-HIV I/II HIV RNA
Human T-lymphotropic virus (HTLV) I/II	Anti-HTLV I/II	Not required
Syphilis	Serologic test for syphilis, every donation	Serologic test for syphilis, every 4 months for donors only
West Nile virus (WNV)[b]	WNV RNA	Not required

[a] The FDA also encourages in-process NAT testing for parvovirus B19 and hepatitis A. HBV NAT testing also is performed on most Source Plasma.
[b] Testing for WNV is recommended in an FDA draft guidance. The FDA is considering recommendations regarding testing for *Trypanosoma cruzi* (Chagas disease).

NAT, of which polymerase chain reaction (PCR) is the most widely used form, does not rely on the detection of antibodies produced by the infected host after exposure, but targets the nucleic acid of the infecting agent. By means of the selection of suitable priming molecules (the so-called primers), the assay is highly specific for the infecting virus (see the *USP* general information chapter *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩). Through several cycles of amplification, the polymerase enzyme can repetitively generate copies of the targeted fragment of the viral nucleic acid, providing an exponential amplification of a very short stretch of the viral deoxyribonucleic acid (DNA) [or ribonucleic acid (RNA)]. The exponential amplification

leads to the generation of many copies of the target molecule and allows the subsequent detection of this virus-specific fragment, even if the original viral load was exceedingly low. This methodology has brought a new degree of safety.

NAT testing, because of its complexity and expense, is difficult to conduct on individual donations. Generally, aliquots from several donations are combined into a single pool, often called a minipool. Testing in pooled format remains more sensitive than serological ELISA screening of individual donations. In addition, the NAT principle circumvents several of the limitations in detecting pathogens by means of serological methods. Pooling can influence overall sensitivity, depending on the pool size and the analytical sensitivity of the NAT assay employed.

In many countries, the maximal load of a pathogen acceptable for a single donation defines the overall NAT sensitivity required. Assays of higher analytical sensitivity can use larger pools, but those of lower analytical sensitivity must test smaller pools in order to comply with regulations. The availability of commercial NAT test kits with defined analytical sensitivity has made minipools up to 512 very common, because these pool sizes, in combination with the analytical sensitivity of the assays used, comply with common regulations on overall sensitivity.

Effective NAT screening requires that the viral load of the plasma pool at the beginning of production be less than the inactivation and/or removal capacity of the process. Differences between the plasma transfusion and fractionation industries have led to different applications of NAT. For individual donations intended for transfusion, where there is no inactivation and/or removal process and where testing is the only method to interdict a contaminated donation, the safety of each individual donation must be ensured by testing with the most sensitive assays possible. Plasma intended for further manufacturing, in contrast, is pooled and serves as the starting material for a multistep process that has built-in pathogen inactivation methodologies. Therefore, NAT screening for plasma for further manufacture is focused on ensuring safe donations and limiting the viral load of the plasma pool to levels less than the known viral inactivation/removal capacity of the inactivation process.

To avoid the loss of large amounts of plasma from a reactive pool, the fractionation industry has implemented a prescreening strategy, the minipool screening concept mentioned earlier. Aliquots of plasma donations are combined to form minipools, and the minipools are tested by NAT. If a minipool is reactive for a virus tested, the individual donation that gave rise to this positive result can be identified and interdicted. The other donations demonstrated to be free of infection can be used for further manufacturing. The donations are then combined into a production pool, a sample of which is subjected to NAT testing as required by regulations.

As indicated, the NAT test portfolio is not uniform and depends on the intended use of the donation and the regulatory environment. Although screening for HCV RNA is done in most countries, screening for HIV is not universally required. NAT detection of HBV is used mainly for plasma for manufacturing. Screening for B19 virus or hepatitis A virus (HAV) is performed only on plasma for manufacturing. HBV screening using NAT detection is more widespread in European and Asian countries than in the United States.

Donor Deferral

A donor may be deferred from further donation as a result of answers provided on the donor history questionnaire, counseling of donors for reasons for deferral, a medical examination performed at the time of donation, or positive tests for infectious diseases. These processes ensure both the eligibility of the donor and the suitability of the donation. In the event that either is not acceptable, standard processes permanently remove the donor and interdict unused units previously donated. The donor registries mentioned earlier are one means of ensuring that a donor deferred at one center cannot donate elsewhere.

Quarantine and Inventory Hold

Each individual unit of plasma, whether for transfusion or further manufacture, is held in quarantine until all the required tests have been completed. If all required tests have been performed and found acceptable, the unit can be released; if not, the unit must be destroyed. The plasma industry has voluntarily implemented the inventory hold protocol (also discussed in the previous section *Donor Screening*) for plasma for further manufacture. According to inventory hold requirements, during a 60-day hold period an individual plasma donation cannot be used for further manufacture. The rationale for the hold is that donors who have been recently infected with a pathogen may not have developed levels of antibody at the time of donation, thereby donating an infectious unit despite negative disease tests. The hold provides sufficient time for an infectious donor to develop levels of antibody that will be detected during a subsequent donation if the plasma was intended for transfusion. The 60-day hold also reduces the chance of releasing an infectious unit into the manufacturing process.

The introduction of NAT may have decreased the need for inventory hold, because NAT targets the infecting virus directly and thus does not rely on the time-delayed production of antibodies. Because NAT cannot detect all viruses and because even NAT has a certain (although very low) limit of detection, inventory hold is still of value and thus remains in place in the plasma fractionation industry.

Investigation

Each plasma donation must be traceable from donation to ultimate disposition in order to minimize the potential transmission of an infectious agent. Traceability encompasses all data concerning donation site, donor identifying information, test results, and data regarding transport, storage, and consignee(s).

Look-back is a process to identify and interdict (quarantine) previous donations from a donor who, at a subsequent donation event, has been found to be (1) infected with a transmissible agent or (2) unsuitable for donating plasma because of history, physical examination, or post-donation information. Although look-back strategies are similar in most countries, specific procedures may vary.

QUALITY SYSTEMS

The intent of this section is to outline the general principles and regulations that are the basis of quality systems relating to plasma collection. U.S. collection centers must follow cGMPs that originate in CFR and are elaborated in FDA regulations, guidance documents, and industry standards.

The GMP regulations specifically governing plasma are found in 21 CFR 600, Biological Products: General; and 606, Current Good Manufacturing Practice (cGMP) for Blood and Blood Components. More general cGMP regulations are found in 21 CFR 210, Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or General Holding of Drugs, General; and 21 CFR 211, Current Good Manufacturing Practice for Finished Pharmaceuticals. Although quality systems regulations are part of 21 CFR for medical devices, they have been extended to other manufacturing as part of the "c" in cGMP and FDA Guidance documents.[4]

The quality system is divided into four major parts: management responsibility, resources, manufacturing operations, and evaluation activities. These are the foundation of the five manufacturing systems: production, facilities and equipment, laboratory control, materials, and packaging and labeling. The procedures of each system are designed to allow operations that facilitate implementation of cGMP requirements. In many instances, these requirements relate to providing facilities and expertise to achieve the requirements for the Five-Layer Safety Net (donor screening, blood testing, donor deferral, quarantine, and investigation), discussed above.

Management responsibilities include providing leadership; building a quality system for the organization that meets requirements; establishing policies, objectives, and plans; and reviewing the quality systems with defined frequency. Resources include having sufficient resources for operational activities, personnel development plans, adequate facilities, and suitable equipment; and controlling outsourced operations. Manufacturing includes designing, developing, and documenting product and processes, performing and monitoring operations, and addressing nonconformities. Evaluation activities include analyzing data for trends, conducting internal audits, and initiating corrective and preventive actions.

A number of required routine activities related to collection and release of plasma or normal recovered plasma from whole blood are linked to both cGMP guidelines and quality systems. These include the requirement for having SOPs to cover all aspects of collection, testing, and release. It is also necessary to validate equipment and systems used by the collection center, including temperature-controlled areas, laboratory equipment, water systems, and computer systems. The design and operation of the facility must be adequate to perform the tasks at hand and prevent cross-contamination. Plasma collection facilities must have an adequate number of knowledgeable and trained staff as well as procedures for acceptance and release of raw materials. To the extent possible, collection facilities must adhere to the GMP requirements for a pharmaceutical manufacturing facility.

GLOSSARY

Apheresis—A method of obtaining one or more blood components by machine processing of whole blood; the residual components of the blood are returned to the donor during or at the end of the process.

Blood component—A constituent of human blood: red cells, white cells, platelets, or plasma.

Blood establishment—Any structure or organization responsible for any aspect of the collection and testing of human blood or blood components, whatever their intended purpose, and their processing, storage, and distribution. Hospital transfusion services engaged only in compatibility testing and transfusion of blood and blood products are not included within the definition of blood establishment.

Blood product—Any therapeutic product derived from human blood or plasma.

Center—Collection site or location where blood or plasma is collected (and also may be processed and stored). *Center* is also applicable to a testing *Site* (see entry in this glossary).

Cryoprecipitate—A plasma component prepared from fresh-frozen plasma by freeze-thaw precipitation of proteins and subsequent concentration and resuspension of the precipitated proteins in a small volume of the plasma.

Deferral—Temporary or permanent suspension of the eligibility of an individual to donate blood or blood components.

Distribution—The act of delivery of blood and blood components to other blood establishments, hospital blood banks, and manufacturers of blood products.

[4] FDA. Guidance for Industry: Quality Systems Approach to Pharmaceutical cGMP Regulations. 2006. Available at www.fda.gov/cber/gdlns/qualsystem.htm.

Manufacturing pool—A combination of a specified number of plasma donations used as the first step in the manufacture of plasma derivatives.

Donation minipool—A combination of a small number of units or samples representative of donations used for pretesting prior to pooling units for manufacture.

Quarantine—The physical isolation of blood components or incoming materials/reagents over a variable period of time while awaiting acceptance, issuance, or rejection of the blood components or incoming material/reagents.

Site—Any location at which a blood establishment carries out blood collection, not including any location not owned or managed by the blood establishment at which blood is collected or any mobile blood collection unit.

Validation—The establishment of documented and objective evidence that the particular requirements for a specific intended use can be consistently fulfilled.

ABBREVIATIONS

AABB	American Association of Blood Banks
AIDS	Acquired immune deficiency syndrome
CFR	Code of Federal Regulations
CJD	Creutzfeldt-Jakob disease
CMV	Cytomegalovirus
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EIA	Enzyme immunoassay
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFP	Fresh-frozen plasma
FP24	Plasma frozen within 24 hours after phlebotomy
cGMP	Current Good Manufacturing Practice
HAV	Hepatitis A virus
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCT	Hematocrit
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-lymphotropic virus
IgA, IgG, IgM	Immunoglobulins A, G, and M, respectively
IQPP	International Quality Plasma Program
IU	International Unit
NAT	Nucleic acid amplification technology
NDDR	National Donor Deferral Registry
PCR	Polymerase chain reaction
PPTA	Plasma Protein Therapeutics Association
PRP	Platelet-rich plasma
RNA	Ribonucleic acid
SARS	Severe acute respiratory syndrome
TEP	Therapeutic exchange plasma
WBDP	Whole blood-derived plasma
WNV	West Nile virus

APPENDICES

Appendix 1

NOTE—The collection, processing, and uses of plasma have generated a large number of terms and definitions that reflect the diversity of operations. In addition to the FDA standards and terms, industrywide voluntary standards are discussed in the *Plasma Safety Considerations* section.

Advisory Note: These terms are not meant as regulatory definitions, because plasma term definitions can vary from region to region and among industry sectors. The reader is advised to consult with regulatory authorities responsible for the region and industry sector. Often specific process variables must be considered.

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions

Plasma Type and Agency or Agency Type	Specification
Recovered plasma	
CFR	Plasma derived from single units of whole blood as a by-product in the preparation of blood components from whole blood collection and intended for further manufacturing. Compliance Policy Guides Manual (CPG 7134.12), Sec. 230.100.
AABB	Plasma for use in manufacturing and prepared from allogenic donations. Plasma selected for manufacture that has been collected from whole blood or apheresis plasma collected for transfusion that has expired.
Inter-region, Inter-sector	Plasma separated from whole blood most often by manual centrifugation or by apheresis. The priority for the blood collected is usually for the production of red blood cells. However, the plasma can be suitable for further manufacture of biotherapeutics and transfusion. The time from collection to freezing can vary depending on the distance of collection and processing sites. Volunteer donors typically are used.
Source Plasma	
CFR	Fluid portion of human blood collected by plasmapheresis and intended as source material for further manufacturing use (21 CFR 640.60).
Inter-region, Inter-sector	Plasma separated from whole blood by plasmapheresis where the cellular components can be returned to the donor. The priority for the plasma usually is for further manufacture of biotherapeutic products. However, the plasma can be suitable for transfusion. It is rapidly frozen after collection.
Fresh-frozen plasma (FFP)	
CFR	Fresh-frozen plasma shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and minimal manipulation of the donor's tissue. The plasma shall be separated from the red blood cells and placed in a freezer within 8 hours or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system and stored at −18° or colder [21 CFR 640.34(b)].
AABB	Plasma separated from the blood of an individual donor and placed at −18° or colder within 6 to 8 hours of collection from the donor or within the timeframe specified by the manufacturer's instructions.
Inter-region, Inter-sector	Plasma that is collected and frozen quickly after preparation. Transfusion is the primary intended use. However, FFP can be suitable for further manufacture of biotherapeutic products.
Council of Europe	A component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain labile coagulation factors in a functional state (Chapter 21).
UK	Supernatant plasma separated from a whole blood donation or plasma collected by apheresis, frozen, and stored.
Australia	Plasma, Fresh Frozen is a component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain the labile coagulation factors in a functional state. If prepared from whole blood, it should preferably be recovered within 6 hours, and not more than 18 hours after collection if the unit has been refrigerated. Plasma may also be collected up to 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°. Separated plasma must be frozen to below −30° within one hour. Freezing of plasma collected by apheresis, as above, must commence within 6 hours of collection or within 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°.
Concurrent plasma	
Inter-region, Inter-sector	Plasma collected concurrently with cellular components. Concurrent plasma may be suitable for transfusion or for further manufacture of biotherapeutics.
Applicant Donor	
Inter-region, Inter-sector	Source Plasma obtained during the first collection from a new donor. The plasma is reserved for testing, and any remainder or products derived from the remainder are not allowed for use in humans or are quarantined until the donor passes appropriate tests and returns for a second donation which also clears testing. At that time, both collections are reclassed as "Qualified".
Platelet-rich plasma (PRP)	

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions *(Continued)*

Plasma Type and Agency or Agency Type	Specification
CFR	PRP shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and manipulation of the donor's tissue. The plasma shall be separated from the red blood cells by centrifugation within 4 hours after completion of the phlebotomy or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system. The time and speed of the centrifugation shall have been shown to produce a product with at least 250,000 platelets per µL. The plasma shall be stored at a temperature between 20° and 24° immediately after filling the final container. A gentle and continuous agitation of the product shall be maintained throughout the storage period if stored at a temperature of 20° to 24° [21CFR 640.34(d)].
Inter-region, Inter-sector	Plasma that is a product of the first centrifugation of blood where it is separated from red cells. Platelets are fractionated into the plasma layer.
Platelet-poor plasma	
Inter-region, Inter-sector	Plasma that is further purified from platelets by a second centrifugation of PRP.
Cryo-poor plasma	
CFR	Plasma that remains after both platelets and cryoprecipitated AHF have been removed may be labeled "Plasma, Cryoprecipitate Reduced" [21CFR 640.34(e)(2)].
AABB	Plasma Cryoprecipitate Reduced; Fresh-frozen Plasma from which cryoprecipitate has been removed.
Inter-region, Inter-sector	Plasma that has been thawed by maintaining the temperature just above freezing (usually 4°). A large portion of certain plasma proteins (e.g., FVIII, cryoprecipitate, fibrinogen, fibronectin, or FXIII) has been precipitated from the plasma.
Council of Europe	Plasma, Fresh-Frozen, Cryoprecipitate-Depleted (Chapter 23). A component prepared from plasma by the removal of cryoprecipitate.
UK	Plasma cryoprecipitate-depleted for transfusion means a plasma component prepared from a unit of plasma, fresh-frozen. It comprises the residual portion after the cryoprecipitate has been removed.
Australia	Plasma, Fresh Frozen, Cryoprecipitate-Depleted is a component prepared from Fresh Frozen Plasma by the removal of cryoprecipitate. The content of albumin, immunoglobulin, and most clotting factors is maintained, but the levels of Factors V and VIII and fibrinogen are reduced. It can be stored for up to 36 months at below −25°.
Cryo-rich plasma	
Inter-region, Inter-sector	Plasma that has been thawed by gentle heat input (e.g., in a 37° water bath) where the cryoprecipitate remains dissolved.
Plasma for labile products	
Inter-region, Inter-sector	Plasma that has been collected and best maintains the activity and integrity of labile plasma proteins as exemplified by clotting Factor VIII. Generally the time from collection through processing to freezing is rapid.
Plasma for stable products	
Inter-region, Inter-sector	Plasma that has been collected where conditions for preservation of labile products was not achieved, however conditions were sufficiently moderate so relatively stable products like IgG and albumin would not be impacted.
Less than 6-hour plasma	
Inter-region, Inter-sector	This is generally recovered plasma that has been collected, processed, and frozen prior to 6 hours after collection. This plasma generally is considered to be acceptable for the production of labile products.
6- to 12-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 6 hours and less than 12 hours after collection. This plasma is generally considered to be acceptable for the production of labile products but is inferior to less than 6-hour plasma for this purpose.
12- to 24-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 12 hours and less than 24 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose.
Less than 12-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen less than 12 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose.

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions *(Continued)*

Plasma Type and Agency or Agency Type	Specification
More than 24-hour plasma	
Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 24 hours after collection and usually less than 72 hours after collection. This plasma generally is not acceptable for the production of labile products.
Pooled plasma	
Inter-region, Inter-sector	Plasma that has been pooled for manufacturing from several donors. Some plasma pools for further manufacture of biotherapeutic products can be derived from several hundred to a few thousand donors.
Single-donor plasma	
Inter-region, Inter-sector	Plasma derived from a single donor. It can be a single unit or a pool of several units derived from multiple collections from the same donor.
Hyperimmune plasma	
Inter-region, Inter-sector	Plasma derived from donors with high titers to specific disease agents. Titers are elevated in these donors mostly as a result of immunization with a vaccine (e.g., hepatitis B, tetanus, or rabies) or exposure to disease agents (e.g., HCV or SARS). Hyperimmune plasma usually is intended for the preparation of IgG to provide passive immunity against target disease agents.
S/D plasma	
Inter-region, Inter-sector	Plasma that has been treated with solvent/detergent, an inactivation method effective against envelope virus disease agents (e.g., HIV, HBV, or HCV). Some plasma protein components are inactivated or damaged by the process (e.g., alpha-1 proteinase inhibitor, Protein S, anti-plasmin, or FVIII).
EU (PharmEuropa)	Human Plasma Pooled and Treated for Virus Inactivation is a frozen or freeze-dried, sterile, nonpyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion. The human plasma used complies with the monograph on Human Plasma for Fractionation.
Therapeutic exchange plasma (TEP)	
Inter-region, Inter-sector	Similar to Source Plasma in its collection. However, the donors are patients who are having their plasma replaced with electrolytes, protein solutions, or plasma from another donor. The objective usually is to remove disease elements from the patient's plasma. Generally, TEP is not advisable for further manufacture of biotherapeutic products. However, there may be cases where a specialty product may propose a specific TEP as a source material.
Quarantine plasma	
Inter-region, Inter-sector	Plasma that has been collected and not had initial testing completed and/or stored as part of a controlled donor program. The donor is retested for disease agents (e.g., 6 months after collection). If the donor again is negative for the tested disease agents, then the plasma is released for use for further manufacture and/or use in humans.
Quarantine residual plasma	
Inter-region, Inter-sector	Plasma that has been collected and stored as part of a controlled donor program. The donor was not retested for disease agents (e.g., 6 months after collection). An example would be that the donor did not return to the collection facility to permit the later test. Quarantine Residual Plasma is not recommended for further manufacture and/or use in humans.
Salvaged plasma	
Inter-region, Inter-sector	Plasma that has experienced a storage or transport temperature deviation but may still be useful for the preparation of nonlabile products such as albumin or IgG.
Plasma for fractionation (Processing requirements)	
CFR	Placed in a freezer within 8 hours and stored at −18° or colder.

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions *(Continued)*

Plasma Type and Agency or Agency Type	Specification
Australia	The liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products. When the plasma is intended for the recovery of proteins that are labile in plasma, it is frozen rapidly to −25° or below within 24 hours of collection. For the recovery of nonlabile proteins, the plasma should be frozen to −20° or below as soon as possible and at the latest within 72 hours of collection. Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below −20°. For accidental reasons, the storage temperature may rise to above −20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds −20° does not exceed 72 hours; the temperature does not exceed −15° on morethan one occasion; the temperature at no time exceeds −5°.
EU (PharmEuropa)	Human plasma for fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products. When obtained by plasmapheresis or from whole blood (after separation from cellular elements), plasma intended for the recovery of proteins that are labile in plasma is frozen within 24 hours of collection by cooling rapidly in conditions validated to ensure that a temperature of −25° or below is attained at the core of each plasma unit within 12 hours after it is placed in the freezing apparatus. When obtained by plasmapheresis, plasma intended solely for the recovery of proteins that are not labile in plasma is frozen by cooling rapidly in a chamber at −20° or below as soon as possible and at the latest within 24 hours of collection. When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and is frozen in a chamber at −20° or below as soon as possible and at the latest within 72 hours of collection. Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below −20°. For accidental reasons, the storage temperature may rise to above −20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds −20° does not exceed 72 hours; the temperature does not exceed −15° on more than one occasion; the temperature at no time exceeds −5°.
Plasma frozen within 24 hours after phlebotomy	
CFR	Plasma manufactured from whole blood should be frozen within 24 hours after phlebotomy. Blood component must be labeled "Plasma Frozen Within 24 Hours after Phlebotomy."

Appendix 2: Donor Criteria

Criterion	Region: United States	United Kingdom	European Union	Australia
General criteria for blood donation				
Appearance	Donor should appear to be in good health (AABB)	Donor should be in good health	Only donors in good health accepted	Nothing specific noted
Underlying medical conditions	Most serious medical conditions are grounds for deferral under United States, EU, and UK guidelines. FDA guidelines require deferral only if a person has used bovine insulin manufactured from UK cattle. However, AABB guidelines require deferral for cancer, heart, liver, or lung disease, and bleeding tendency unless approved by a medical director. Cancer and cardiac disease and diabetes treated with insulin require permanent deferral under EU and UK guidelines			Most serious medical conditions are grounds for deferral. Cancer: accepted 5 years of remission. Cardiac disease: varies depending on clinical condition. Diabetes: acceptable if controlled.
Age	≥16 or conform to applicable state law (AABB, whole blood)	Between 18 and 65; donation at 17 permitted if in accord with national legislation; first-time donors >60 only if permitted by physician	Whole blood: between 17 and 65; no first-time donors >60 Apheresis: first-time donors between 18 and 60; may donate up to age 65	Whole blood: can start at 16–17 with consent of parents and continue to 80, but medical review required at >70. Apheresis: accept new donors 18–65, with medical evaluation required when >60. Existing donors require annual medical review if >65.
Weight	None stated: no more than 10.5 mL/kg may be withdrawn (AABB) FDA requires Source Plasma donors to weigh at least 110 lb	Whole blood: ≥50 kg Apheresis: no specific weight requirement	Whole blood: ≥50 kg Apheresis: ≥50 kg	<45 kg—defer. Medical opinion required for unexplained weight loss
Blood pressure	Systolic ≤180 mm Hg Diastolic ≤100 mm Hg (AABB)	Systolic ≤180 mm Hg Diastolic ≤100 mm Hg	No specified blood pressure parameters; donors with high blood pressure may donate provided (1) they have not suffered any complications caused by high blood pressure, (2) they are taking only beta blockers and/or diuretics, and (3) their disease is stable, as determined by qualified medical personnel.	Acceptable ranges: Systolic 90–180 mm Hg. Diastolic 60–90 mm Hg. Hypertension 180–100: defer. Hypotension 90–60: defer.
Pulse	Between 50 and 100 beats per minute and regular; lower pulses acceptable at discretion of physician (AABB)	Between 50 and 100 beats per minute and regular	No specific pulse rate parameters stated	Regular pulse between 50 and 100: accept Pulse between 40 and 49: accept if donor is physically fit and not on medication
Temperature	≤37.5° taken orally at time of donation (AABB)	Donors who have had a temperature of ≥38° may not donate for 2 weeks	Donors who have had a temperature of ≥38° or flu-like symptoms may not donate for 2 weeks	No requirement
HgB/HCT	≥125 g/L or HCT 38%	Males: ≥135 g/L Females: ≥125 g/L	Males: ≥135 g/L Females: ≥125 g/L	Whole blood: females, 120–165 g/L; males, 130–185 g/L. Apheresis: females, 115–165 g/L; males, 125–185 g/L. If high, defer. If low, defer for 6 months and test for ferritin.

Appendix 2: Donor Criteria *(Continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Skin examination	Free of infectious skin disease at site of phlebotomy; no skin punctures or scars indicative of addiction to self-injected narcotics (CFR) Free of infectious diseases (AABB)	Skin at venipuncture site should be clear of lesions, including eczema	There should be no skin disease at venipuncture site	Avoid venesection where there is evidence of inflammation or infection
Pregnancy	Defer for 6 weeks after delivery (AABB)	Defer 6 months after delivery	Defer for 1 week for every completed week of pregnancy	Current: defer 9 months from estimated date of confinement. After third-trimester delivery: defer 9 months. Miscarriage or termination: defer 3, 6, or 9 months, respectively for 1st, 2nd, and 3rd trimester.
Underlying medical conditions that require deferral and that do not pose a risk of transfusion-transmissible infection				
Cancer	Permanent deferral unless deemed suitable by medical director (AABB)	Permanent deferral although physician may make exceptions. Permitted after cervical cancer or basal cell carcinoma if successfully treated	Malignant neoplasms, including leukemias and myeloproliferative disorders, are cause for permanent deferral; exceptions may be made for certain conditions after successful therapy	Permanent deferral for haematological malignancies. Skin cancer–basal–cell carcinoma: accept. Other cancers: defer 5 years after completion of treatment
Cardiac disease	Free of major organ disease (heart, liver, and lungs) unless deemed suitable by medical director (AABB)	Permanent deferral for persons with a history of heart disease, especially coronary disease, angina pectoris, severe cardiac arrhythmia, arterial thrombosis, or recurrent venous thrombosis. 2-year deferral for rheumatic heart disease with no evidence of chronic disease	Permanent deferral for persons with active or past serious cardiovascular disease, except congenital abnormalities with complete cure	Permanent deferral for arrhythmias, endocarditis, ischaemic heart disease, heart surgery, myocardial disease. Accept: congenital heart disease if surgically corrected. Heart murmurs: accept, subject to medical opinion. Accept after full recovery: pericardial disease, rheumatic heart disease.
Cerebrovascular diseases	No specific guideline other than that the donor must be free of major organ disease (AABB)	Permanent deferral for history of cerebrovascular diseases	Permanent deferral for donors with a history of serious CNS disease	Permanent deferral
Epilepsy	No deferral	Must be free of epileptic attack for 3 years and have been taken off all medication	Permanent deferral unless at least 3 years have elapsed since the date that donor last took anticonvulsant medication and there has been no recurrence of symptoms	Defer for 2 years from last seizure
Gastrointestinal disease	No specific deferral; free of major organ disease unless deemed suitable by medical director (AABB)	No specific deferral	Deferral (not noted to be a permanent deferral) for disease that renders the individual liable to impaired iron absorption or blood loss	Ulcers: defer indefinitely

Appendix 2: Donor Criteria *(Continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Genitourinary and renal disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Five-year deferral after complete recovery from acute glomerulonephritis	Permanent deferral for donor with serious genitourinary or renal disease	Permanent deferral: chronic pyelonephritis, chronic kidney infection, chronic dialysis. Accept if resolved: haematuria, acute kidney infection. Urinary catheter present: plasma only for fractionation if underlying condition acceptable. Acute dialysis: defer for 12 months. Acute glomerulonephritis: defer 5 years after recovery.
Diabetes	No specific deferral except that receipt of bovine insulin manufactured in the UK requires permanent deferral (FDA). Free of major organ disease unless deemed suitable by medical director	Permanent deferral if insulin therapy required	Permanent deferral for donors on insulin treatment	Permanent deferral if diabetes-associated complications present Accept if disease is controlled, even if patient is taking insulin
Respiratory disease	Free of acute respiratory disease (CFR). Free of major organ disease (lungs) unless deemed suitable by medical director (AABB)	Permanent deferral for chronic bronchitis; common cold acceptable	Permanent deferral for serious disease	Permanent deferral: chronic abscess, bronchiectasis, or emphysema with respiratory insufficiency. Accept if mild and controlled: asthma, bronchiectasis without respiratory insufficiency Plasma for fractionation only: chronic bronchitis. Acute bronchitis: defer 2 weeks after recovery and being off antibiotics for 5 days. Pleurisy, pneumonia: defer 4 weeks after recovery. Acute pulmonary embolism: defer 12 months after recovery
Hematologic disorders	Free of abnormal bleeding tendency unless determined suitable by medical director (AABB) Free of major organ disease (cancer) unless deemed suitable by medical director (AABB)	No specific deferral. Donors who are heterozygous for beta thalassemia eligible if HgB values are within normal limits	Permanent deferral for donors with serious hematologic diseases	Permanent deferral: donors with serious hematologic diseases (sickle cell disease, thalassaemia major). Accept: thalassaemia minor. Defer: anaemia Plasma for fractionation only: elliptocytosis, glucose-6-phosphate dehydrogenase (G6PD) deficiency, spherocytosis. Apheresis only permitted for patients with G6PD deficiency
Immunologic disorders	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Permanent deferral for donors with autoimmune disease that affects more than one organ. Defer: documented history of anaphylaxis	Permanent deferral for donors with serious immunologic diseases	Permanent deferral: autoimmune disorders. Accept: If asymptomatic, only one organ system involved, not on immunosuppressive therapy. Accept: Sjogren's syndrome

Appendix 2: Donor Criteria *(Continued)*

Criterion	Region: United States	United Kingdom	European Union	Australia
Metabolic disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	No specific deferral	Permanent deferral for serious metabolic disease	No specific reference
Bone disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Two-year deferral after having been declared cured of osteomyelitis	No specific deferral	No specific reference
Surgery	No specific deferral unless blood was transfused; in which case, a 12-month deferral applies (CFR)	Major surgery requires evaluation of risk for transfusion-transmissible disease	Permanent deferral for persons with history of resection of the stomach. Major surgery requires a 6-month deferral; minor surgery requires a 1-week deferral	Minor (e.g., skin lesions, arthroscopy): defer until recovered. Routine minor (e.g., appendectomy, laparoscopy): defer for 2 months. Major surgery (if donor received autologous blood only): defer 6 months. Neurosurgery; medical assessment required.
Medications that require deferral				
Antibiotics	As defined by medical director (AABB)	Donors treated with any prescribed drug should be deferred for a period consistent with the pharmacokinetic properties of the drug	Defer for 2 weeks from full recovery or 1 week from cessation of antibiotic therapy, whichever is longer	Acute treatment: defer until recovered and off antibiotics for 5 days. Prophylactic: accept plasma only for fractionation. Topical: accept if skin is unbroken and not infected.
Drugs with teratogenic potential	Etretinate: permanent deferral (FDA). Acitretin: 3-year deferral from last dose (FDA). Dutasteride: 6-month deferral from last dose (FDA). Isotretinoin: 1-month deferral from last dose (FDA). Finasteride: 1-month deferral from last dose (FDA).	Donors treated with drugs with proven teratogenic effect should be deferred for a period consistent with the pharmacokinetic properties of the drug	Donors taking drugs that are proven or potential teratogens or who are taking drugs that accumulate in the tissues over long periods of time should not be used as blood donors	Raloxifene (Evista): defer for 6 months after completion of treatment. Acitretin (Neotigason): defer for 3 years after completion of treatment. Etretinate (Tigason): permanent deferral. Finasteride (Proscar): 7 days after completion of treatment. Isotretinoin: defer for 8 weeks after completion of treatment.
Growth hormone from human pituitary glands	Permanent deferral (FDA)			Permanent deferral
Other drugs	No other deferrals by FDA or AABB. Any other deferral at discretion of medical director of blood center (AABB). Warfarin: 7-day deferral for plasma donation (AABB).	Recommended that a list of commonly used drugs with rules for acceptability of donors, approved by the medical staff of the transfusion center, be available.	Other drugs acceptable as long as the underlying condition for which the drug is taken is acceptable.	Most medications that are taken by donors are not harmful to recipients; therefore people taking medications can be acceptable as blood donors. Eligibility is based on the assessment of the underlying condition and specific medication guidelines.
Insulin	Permanent deferral: bovine insulin made in UK (FDA).	Permanent deferral if treated with insulin	Permanent deferral if treated with insulin	Defer if diabetes is poorly controlled
Immunizations				

Appendix 2: Donor Criteria *(Continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Toxoids	No deferral	No deferral	No deferral	
Licensed killed bacterial vaccines	No deferral	No deferral		No deferral
Licensed inactivated viral vaccines	No deferral	No deferral	No deferral except 1-week deferral after hepatitis B vaccination	No deferral except 1-week deferral after hepatitis B vaccination
Unlicensed killed vaccines	1-year deferral (AABB)			Defer 3 months after vaccination
Inactivated rickettsial vaccines	No deferral	No deferral		
Live attenuated bacterial and viral vaccines	4 weeks for varicella and rubella 2 weeks for rubeola, yellow fever, mumps, polio (oral), typhoid (oral) (AABB)	4 weeks	8 weeks	Plasma only for fractionation for 4 weeks after vaccination
Transfusion-transmissible infections that require deferral				
HIV infection/AIDS and sexual partners	Permanent deferral if present or past clinical or laboratory evidence of HIV infection/AIDS: positive EIA with positive or indeterminate confirmatory test; positive NAT test; clinical signs include unexplained weight loss, night sweats, blue or purple spots in mouth or on skin, white spots or unusual sores in the mouth, swollen lymph nodes for more than 1 month, persistent cough or shortness of breath, persistent diarrhea, fever for more than 10 days; sexual partners deferred for 1 year from time of last contact (FDA)	Permanent deferral for donors found to have a confirmed positive marker for HIV. Donors found to have a repeat positive marker for HIV that cannot be confirmed should be informed according to the nationally agreed algorithm.	Donors with HIV I or II must be permanently deferred	Infection: permanent deferral. Relevant symptoms within the last 6 months: defer for 12 months. Sexual contact with HIV-positive partner: defer for 12 months after last sexual contact.
Hepatitis and sexual partners and household contacts	Permanent deferral for the following: History of viral hepatitis after the 11th birthday. Confirmed repeatedly reactive for HBsAg. Positive test for anti-HBc on more than 1 occasion (testing is not required for Source Plasma donors). Present or past laboratory or clinical evidence of infection with HCV. Sexual partners of patients with hepatitis deferred for 1 year from last contact; household contacts of persons with hepatitis B deferred for 1 year from last contact (CFR).	Permanent deferral for donors whose blood gives a positive reaction for the presence of HBsAg and/or anti-HCV. Donors with a history of jaundice or hepatitis may, at the discretion of the appropriate competent medical authority, be accepted as blood donors, provided that an approved test for HBsAg and anti-HCV is negative.	Permanent deferral for hepatitis B and C: donors with history of hepatitis B may donate after 12 months after recovery, provided that all markers are negative or core antibody positive, HBsAg is negative, and anti-HbS ≥100 IU/L; donors with documented current or past infection with hepatitis C are permanently deferred; donors with hepatitis A are deferred for 12 months.	Hepatitis B acute or past infection: defer for 12 months after recovery, then perform hepatitis testing. Hepatitis B chronic carrier: permanent deferral. Hepatitis B contact, sexual, mucosal, household: defer for 12 months from last exposure unless immune. Hepatitis B other contact: accept. Hepatitis C positive past infection: permanent deferral. Hepatitis C contact, sexual, mucosal, household: defer for 12 months from last exposure; other contact: accept.

Appendix 2: Donor Criteria *(Continued)*

Criterion	United States	United Kingdom	European Union	Australia
	Region			
HTLV	Present or past clinical or laboratory evidence of infection with HTLV I/II (positive EIA on 2 occasions). [HTLV not tested in Source Plasma donors] (FDA)	Permanent deferral for carriers of HTLV I/II	Permanent deferral for donors with HTLV I/II	Infection: permanent deferral. Repeat reactive status: plasma only for fractionation. Sexual contact: defer for 12 months after last contact. Household contact: accept.
West Nile virus	Donors with symptoms suspicious of or actual diagnosis of WNV deferred for 120 days. Donor testing positive on WNV NAT deferred for 120 days. Donor who develops symptoms of WNV within 2 weeks of donation should be deferred for 120 days. Donors implicated in possible transfusion-transmitted WNV infection should be deferred for 120 days (FDA).	Defer for 28 days after donor leaves an area with ongoing transmission to humans.	Defer for 6 months if donor was in area endemic for WNV and was diagnosed with or had symptoms consistent with WNV. Defer for 28 days after donor returns from endemic area, provided donor has no symptoms of WNV.	Infection: defer for 3 months after full recovery. Area exposure: plasma only for fractionation for 8 weeks after leaving risk area.
Chagas disease and babesiosis	Permanent deferral for history of Chagas disease; current FDA regulations do not require testing for Chagas disease. However, although not required, most facilities collecting blood for transfusion perform EIA test for Chagas and permanently defer following positive test. (AABB) FDA probably will not require antibody screening for fractionated or recovered plasma used for further manufacture. FDA has granted exemptions and permitted the collection and distribution of Source Plasma for further manufacture into noninjectable products from a donor known to have Chagas disease (CFR). Permanent deferral for history of babesiosis (AABB)	Permanent deferral for individuals with Chagas disease or history of Chagas disease; blood of persons who were born or have been transfused in areas where the disease is endemic should be used only for plasma fractionation products unless a validated test for infection is negative	Permanent deferral for individuals with Chagas disease. Individuals in the following categories may donate 6 months after leaving an endemic area, provided that a validated test for Chagas disease is negative (if a validated test is positive or not performed, the donor is permanently deferred): born in South or Central America, mother born in South or Central America, transfused in South or Central America; lived or worked in a rural subsistence community in South or Central America for 4 weeks or more	Infection: permanent deferral. Contact: accept. Chagas disease area resident: plasma only for fractionation, permanently. Chagas disease area visitor: plasma only for fractionation for 12 months after leaving endemic area

Appendix 2: Donor Criteria *(Continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Creutzfeld-Jakob disease (CJD) and variant CJD	Permanent deferral if donor: has diagnosis of CJD or nvCJD; has a blood relative diagnosed with CJD; received dura mater graft; received human-derived pituitary growth hormone; received bovine insulin made in UK; spent a cumulative 3 months in UK between 1980 and 1996; received a blood transfusion in the UK at any time since 1980; spent 6 months between 1980 and 1990 on a US military base in Northern Europe; spent 6 months between 1980 and 1996 on a US military base elsewhere in Europe; spent a cumulative 5 years in Europe (Source Plasma donors are not deferred for the latter) (FDA)	Permanent deferral if donor: treated with extracts derived from human pituitary glands; has been recipient of dura mater or corneal graft; has a family risk of CJD or any other TSE. For vCJD: Member states should determine on the basis of the prevalence of BSE within individual countries, of the endogenous exposure of the population to bovine products imported from countries with a high BSE prevalence, and of the incidence of cases of vCJD, what precautionary measures they may need to take to minimize the risk of transmission of vCJD via blood transfusion	Permanent deferral if donor: Diagnosed with CJD, vCJD, or any other prion-associated disease; has family risk of CJD; at increased risk from surgery, transfusion, or transplant of tissues or organs; received a dura mater graft; received a corneal, scleral, or ocular graft; received human-derived pituitary extract; received a blood transfusion in UK since 1980; received intravenous immunoglobulin (IVIg) of UK origin; donated unit of blood implicated in possible case of transfusion-related vCJD. Additionally, all plasma from British donors cannot be used for fractionation	Permanent deferral if diagnosed with any prion-related disease. Permanent deferral if donors have spent a cumulative time of 6 months in England, Wales, Scotland, Northern Ireland, the Channel Islands, or the Isle of Man between 1 January 1980 and 31 December 1996. Permanent deferral if donors received a blood transfusion or blood products in England, Wales, Scotland, Northern Ireland, the Channel Islands, or the Isle of Man from 1 January 1980 onwards unless the blood products were processed plasma products and were given after 31 December 2001. Permanent deferral if the donor had ear surgery performed between 1972 and 1989 and dura mater was used. Permanent deferral for donors who received human-derived pituitary growth and gonadotrophic fertility hormones prior to 1986.
Visceral leishmaniasis (Whole Blood)	Donors who have been to Iraq are deferred for 1 year. Permanent deferral for signs and symptoms of visceral leishmaniasis (FDA)	No specific deferral	Permanent deferral for visceral leishmaniasis	Cutaneous: plasma only for fractionation, permanently Visceral: permanent deferral Contact: accept.
Medical conditions and behaviors that place an individual at risk for a transfusion-transmissible infection and require deferral.				
Xenotransplant	No current deferral required for blood donation, but draft regulations for tissue and organs require permanent deferral.	Permanent deferral	Permanent deferral	Permanent deferral
Blood transfusion organ and tissue transplant; treatment with plasma-derived clotting factor concentrates	One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact	Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months	Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant	Blood transfusion homologous: defer 12 months Coagulation factor, blood derived, short term: defer 12 months from last treatment. Coagulation factor, blood derived, continuous: permanent deferral. Human tissue recipients: Organ/haematological: permanent deferral. Homologous, bone, tendon, skin: accept. Collagen: accept. Corneal: permanent deferral for iatrogenic cCJD risk.

Appendix 2: Donor Criteria *(Continued)*

	Region			
Criterion	**United States**	**United Kingdom**	**European Union**	**Australia**
Surgery or use of endoscope with biopsy	No specific deferral criteria; general health and transfusion criteria apply	Six-month deferral for major surgery; stomach resection requires permanent deferral; 1-week deferral for minor surgery; 6-month deferral for endoscope with biopsy; if NAT test for hepatitis C is negative, may donate after 4 months	Six-month deferral for major surgery or procedure using an endoscope; 1-week deferral for minor surgery	Defer 6 months
Nonsterile skin penetration or mucous membrane exposure to blood or body fluids not the donor's own	Twelve-month deferral	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months	Twelve-month deferral	Defer 12 months
Acupuncture, tattoo, body piercing, etc.	Twelve-month deferral unless performed by a state-regulated entity, using sterile needles and disposable dyes	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months; exception can be made according to national risk assessment	Twelve-month deferral; 6-month deferral if validated test for hepatitis B core antibody is negative; for acupuncture, no deferral if performed by state-regulated entity	If using single-use items: plasma only for fractionation for 12 months If not single-use or unsure: defer for 12 months
Injection of medications or steroids not prescribed by a physician	Permanent deferral; sexual partner deferred for 1 year	All blood donors should be provided with accurate and updated information about HIV transmission and AIDS so that persons who have unsafe sex practices or other risk behavior exposing them to potential infection will refrain from donating. The information provided may vary among countries according to local epidemiological data.	Permanent deferral if donor ever injected or has been injected with drugs; sexual partner deferred for 1 year	Permanent deferral
Males who have sexual contact with another male	Permanent deferral for sexual contact, even once, since 1977; female sexual partner deferred for 1 year from last contact		Permanent deferral for oral or anal sexual contact even if protection used; female sexual partner deferred for 1 year from last contact.	Defer for 12 months after last sexual contact
Accepted money or drugs or other payment in exchange for sex since 1977	Permanent deferral; sexual partner deferred for 1 year from last contact		Permanent deferral; sexual partner deferred for 1 year from last contact.	Defer for 12 months after last sexual contact
Incarceration for more than 72 hours in the past year	One-year deferral		No deferral	Defer for 12 months after release
Born in or lived in Africa	Permanent deferral if born or lived in countries where HIV I subtype O is endemic (Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, Gabon, Niger, Nigeria); sexual partner deferred for 1 year from last contact unless tested with a test validated to detect Group O		No specific deferral for Africa; malaria rules apply; however, sexual partners of persons who were sexually active in areas where HIV is endemic deferred for 1 year from last contact	No specific deferral: donors who have visited a malaria-endemic area are subject to a plasma-only restriction period of at least 12 months. The restriction period is extended to 3 years if residence has been for 6 continuous months or more within the past 3 years. With a negative malaria test, the restriction period can be reduced to 4 months. Donors who have traveled to an HIV risk area must be asked if they had sexual contact with a resident of that area.

Appendix 3: Disease Testing

Disease Test	United States	European Union
Hepatitis		
HBsAg	Permanent deferral for repeatedly reactive test results (FDA)	*European Pharmacopoeia:* **Human Plasma for Fractionation**
Hepatitis B core antibody	Permanent deferral if reactive on 2 or more separate occasions; permanent deferral if core antibody positive results are coupled with prior or concurrent repeatedly reactive HBsAg test (FDA). Testing is not required for Source Plasma donors	Laboratory tests are carried out for each donation to detect the following viral markers: (1) antibodies against HIV-1 (anti-HIV-1). (2) antibodies against HIV-2 (anti-HIV-2). (3) hepatitis B surface antigen (HBsAg). (4) antibodies against hepatitis C virus (anti-HCV). If a repeat-reactive result is found in any of these tests, the donation is not accepted.
Hepatitis C antibody EIA	Permanent deferral if repeatedly reactive for hepatitis C antibody; may re-enter donor after 6 months if confirmatory test is negative (FDA)	**Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations**
Hepatitis C NAT testing	Permanent deferral if positive on single testing (FDA)	The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations: ABO group (not required for plasma intended only for fractionation). Rh D group (not required for plasma intended only for fractionation). Testing for the following infections is required in donors: Hepatitis B (HBsAg), Hepatitis C (Anti-HCV), HIV 1 and 2 (Anti-HIV 1 and 2). Additional tests may be required for specific components or donors or epidemiological situations. **Blood Directive 2004/33/EC (Annex III): Permanent Deferral Criteria (excerpt):** Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune Hepatitis C HIV 1 or 2
HIV		
HIV NAT	Permanent deferral if positive on single testing (FDA)	

Appendix 3: Disease Testing *(Continued)*

Disease Test	United States	European Union
HIV I or II Antibody (EIA)	Permanent deferral for repeatedly reactive HIV EIA test; may reenter after 6 months if confirmatory test negative (FDA). Testing is not required for Source Plasma donors	***European Pharmacopoeia:*** **Human Plasma for Fractionation** Laboratory tests are carried out for each donation to detect the following viral markers: (1) antibodies against HIV-1 (anti-HIV-1) (2) antibodies against HIV-2 (anti-HIV-2) (3) hepatitis B surface antigen (HBsAg) (4) antibodies against hepatitis C virus (anti-HCV) If a repeat-reactive result is found in any of these tests, the donation is not accepted **Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations** The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations: ABO group (not required for plasma intended only for fractionation) Rh D group (not required for plasma intended only for fractionation) Testing for the following infections are required in donors: Hepatitis B (HBsAg) Hepatitis C (Anti-HCV) HIV 1 or 2 (Anti-HIV 1 and 2) Additional tests may be required for specific components or donors or epidemiological situations. **Blood Directive 2004/33/EC (Annex III), Permanent Deferral Criteria (excerpt)** Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune Hepatitis C HIV 1 or 2

⟨1181⟩ SCANNING ELECTRON MICROSCOPY

INTRODUCTION

Over the last few decades, electron microscopy has become a reliable investigative tool for the study of solid and semi-solid materials. Since the invention of the electron microscope attributed to Max Knoll in 1935 and commercialization in the 1960's by Cambridge Instrument Co. and JEOL, recent improvements in resolution, stability, and specimen accommodation have resulted in a robust array of commercial instrumentation that spans a wide range of capabilities. This chapter provides a review of the common electron microscopy technologies and techniques.

UTILITY OF THE TECHNIQUE

General Description of the Technique

Scanning electron microscopy (SEM) is an electron optical imaging technique that yields both topographic images and elemental information and is used in conjunction with a variety of detection systems and elemental detectors. All electron microscopy systems utilize a high energy, focused electron beam as source illumination with concurrent increase in resolution compared to photon (light) microscopy. The resolution increase is realized by electron beam wavelength being three orders of magnitude smaller than that of visible light. The optics consist of condenser and objective lenses in conjunction with selected apertures. A focused e-beam is rastered over the surface of a specimen, generating a variety of signals from which an image is collected.

Traditional High Vacuum SEM

Traditional SEM systems operate at high vacuum, so specimens must be clean, dry, and able to withstand imaging at high vacuum, up to 10^{-6} torr. Additionally, the specimens must be inherently conductive or coated to conduct the high surface charges. Four general principles of these systems are:

- e-beam generation
- image formation
- specimen preparation
- elemental composition.

Typically, SEM analysis requires only a small amount (10^{-3} to 10^{-12} g, depending on the application) of a solid specimen. The specimen is presented to the collimated e-beam and scanned in a controlled raster pattern. Images, or micrographs, are generated using secondary electron (SE) and/or back-scattered electron (BSE) detection. Specimen detail may be resolved to 10 nm even in the most simple systems. The collimation of the e-beam also yields great depth of field that is evident in a wide dynamic range of magnification, practically spanning five orders of magnitude (5×–100,000×).

The size of the final aperture controls the beam diameter and, accordingly, the image resolution and total current at a specimen. Small apertures are required for high resolution and large apertures provide high current for optimal X-ray emission intensity. In many systems, the objective aperture can be adjusted during use with a sliding or rotating holder. Flexibility in trading resolution for specimen current is also important because specimen characteristics affect these two criteria differently.

Image magnification is controlled by altering the area of the electron beam raster; smaller areas yield higher magnification. An Everhart-Thornley (ET) detector is used for electron detection; the resultant images are most similar to those of reflected light microscopy. An ET detector consists of a Faraday cage and a scintillator disk connected by a light pipe to a photomultiplier tube. The Faraday cage serves three functions: (1) at positive bias it attracts SE; (2) at negative bias it repels SE to enable the ET detector to collect BSE signals alone; and (3) it shields the primary electron (PE) beam from the scintillator potential. Various scintillator coatings are used. For example, phosphorus-based coatings yield intense, high-contrast images. Aluminum-based coatings, although less sensitive, can withstand the high SE flux generated during elemental analyses. Additionally, solid-state detectors provide up to 10 times greater sensitivity for BSE collection. They can be placed at a variety of positions and distances with respect to a specimen and used in conjunction with ET detectors.

Variable Pressure or Environmental Scanning Electron Microscopy (ESEM)

Robust and common variations of the traditional high vacuum system prevail in today's labs. So-called variable pressure, "environmental" or low vacuum systems (LVSEM) appeared in the mid 1980s. These are electron microscope systems operating at a much higher, near ambient pressure state (up to 50 torr) at the specimen site, plus gas over-blanketing (almost any may be used) and temperature control to 1500°C as well.

There are two key advantages for ESEM's: First, non-conducting samples do not require a conductive carbon or metal thin film to be applied. The gas in the sample chamber is ionized by the electrons generated from the beam/sample interaction. These ions will diffuse to the sample surface, thereby dissipating any negative charge buildup. Secondly, if the sample in ques-

tion is prone to outgassing volatile substances such as solvents and water, the near-ambient pressures used in the sample chamber can greatly diminish the likelihood of this occurring.

Scanning Transmission Electron Microscopy

The combination of a traditional transmission electron microscope system with scanning control yields specimen analysis with high micrograph resolution, information at the atomic scale such as atomic structure, chemical information, and interfacial bonding. Image acquisition is conducted in the same manner as SEM; however, use of thin specimens allows collection of transmission images as well. Great increase in resolution is attained by controlling the e-beam probe volume, to atomic dimensions. *Figure 1* shows the lattice image of a ceramic that was obtained using a scanning transmission electron microscopy (STEM) instrument. As with any electron- or ion-beam method, the sample must be able to withstand the electron bombardment. This may not always be the case with pharmaceutical materials, as they can degrade in the e-beam during the analysis.

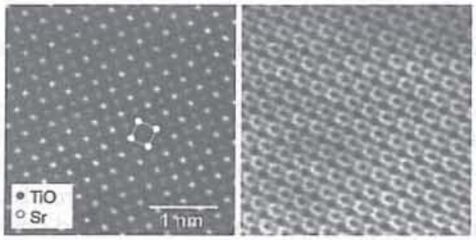


Figure 1. Scanning transmission electron micrograph of an oxide ceramic (perovskite), shown in both dark field (left) and bright field (right) imaging modes. Image courtesy of "... © Carl Hanser Verlag, Muenchen".

ELECTRON BEAM GENERATION

The basis for the formation of an electron image is the generation of an energetic flux of electrons that impinge upon the sample surface. If the beam is too energetic, the sample will be burned as the beam is rastered across the sample's surface. This is an important consideration when analyzing pharmaceutical materials.

Electron guns can be classified into two types: thermionic emitters and field emitters. With the former, the filament is heated under high vacuum, and a large potential is applied. The field that is created, combined with the heated filament, "boils" off electrons that are subsequently focused in the microscope optics. To increase the flux of electrons from thermionic emitters, one must increase the potential that is applied across the filament, which also increases the kinetic energy of the electrons and the likelihood that the sample surface will be burned.

Another method used to produce electronic beams is to apply a large electric field across an electronic emitter. The filament can have a current passing through it or not. Where surface damage is a prime consideration, cold emitters are the best way to obtain a clear image at high magnification without burning the surface or damaging the material. Typically tungsten is used as the electron emitter, as it is strong enough to withstand the mechanical forces. When using a field emission gun, the cathode must be kept at very high vacuum, and no foreign contaminants must be allowed to deposit on the filament. For these reasons, the column must be differentially pumped. Field emission instruments are better suited for the analysis of pharmaceutical compounds, as they produce a very bright beam that is less damaging to the sample. Additionally, field emission electron SEMs have higher spatial resolution, making them better suited for the identification of foreign particulate matter.

Electron Beam–Sample Interactions

Interaction of the electron beam (PE) with the specimen produces a variety of physical phenomena that, when detected, are used to form images and provide elemental information about the specimen. These phenomena include:

- emission of inelastic scattered secondary electrons (SE), proportional to specimen topography
- reflection of elastically scattered back-scattered electrons (BSE), proportional to the atomic number (*Z*) of the specimen
- component X-ray emission (XRE, X in *Figure 2*)
- emission of Auger electrons (AE)
- cathodoluminescence (CL)
- conduction of current (specimen current)
- charging from induced voltages (IV) or adsorbed electrons
- electron transmission
- heat generation
- electromotive forces (see *Figure 2*).

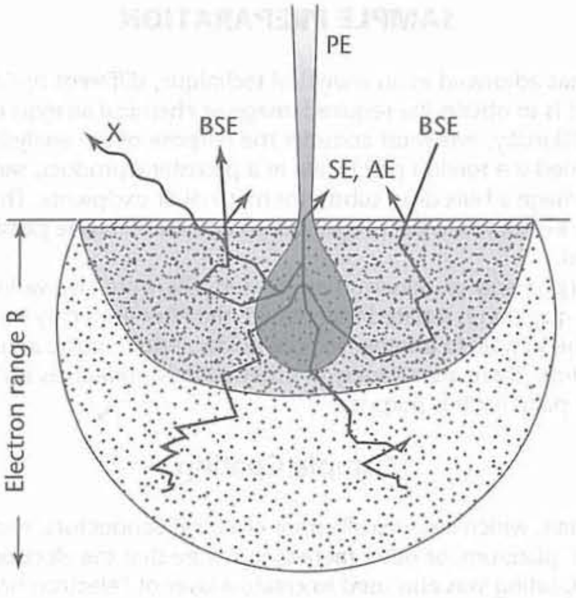


Figure 2. Interaction diagram.

SE are emitted from a specimen surface as the result of inelastic collisions between primary (incident) electrons and the specimen. When the energy imparted to a specimen electron exceeds the work function of a specimen, that electron is emitted as SE. Most secondary electrons have energies of 5–20 eV; electrons in this low-energy range can be efficiently collected, yielding high signal-to-noise images. Because such low-energy electrons can penetrate only short distances through the specimen, SE originate from within 2–30 nm of the surface and generate highly resolved images. The actual e-beam penetration depth is dependent on e-beam accelerating voltage, specimen elemental composition, specimen density, and specimen mounting angle. Excitation volumes of 0.5–5 µm in diameter (dark gray) are typical for a wide range of materials from metallic to biological.

Back-scattered electrons are primaries that have been reflected from the specimen. The primary electrons can undergo multiple collisions prior to exiting from the specimen; therefore, BSE have energies over a broad range and emerge from relatively deep specimen penetration (≈0.1–5 µm) (see *Figure 3*).

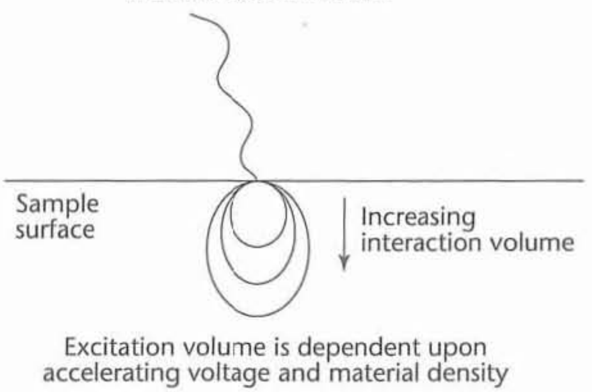


Figure 3. Bulk penetration.

These high-energy (15–25 keV) BSE are collected less efficiently than SE, and they yield images with poorer resolution. The efficiency of BSE reflection is a function of the atomic number (*Z*) of the specimen atoms; thus, the contrast of BSE images depends on elemental composition. The penetration depth of all electrons is affected by elemental composition, specimen density, specimen tilt, and incident beam energy (accelerating voltage). There are a number of software algorithms to correct for these influences, when known such as ZAF, Bence-Albee, etc. For example, the SE images of sodium phosphate and zinc phosphate crystals are quite similar. However, the heavier nuclei of the zinc species produce more efficient BSE reflection, and BSE images will reveal higher contrast. BSE images of heavy- versus light-element phases, or mixtures of species, show dramatic contrast differences that are representative of elemental heterogeneity. Elemental mapping by energy dispersive X-ray spectroscopy (EDS), discussed later, can provide complementary information.

SAMPLE PREPARATION

As scanning electron microscopy has advanced as an analytical technique, different options have become available for sample preparation. Ideally, the objective is to obtain the required image or chemical analysis of the material to be examined with as little intervention as possible. Additionally, one must consider the purpose of the analysis and the amount of material that is available. If the material to be examined is a foreign particulate in a parenteral product, sample preparation could be quite different than if one is intending to image a bulk drug substance material or excipients. The decision tree (see *Figure 4*) below represents a simplified version of the available sample preparation method and is one possible construct to determine the appropriate sample preparation method.

For example, in addition to metal (primarily aluminum) sample stubs there are also various metal meshes that can be used to support samples. If the particle in question is isolated from liquid, the filter itself may serve as a suitable substrate for the powder without having to transfer the sample to another sample holder. Additionally, adhesive carbon tabs are a common means to secure the sample to the stub. There are a variety of other adhesive materials such as double-sided copper, silver and aluminum tape, or carbon and silver paint for this purpose.

Sample Coating

Historically, pharmaceutical materials, which are typically poor electrical conductors, had to be coated with a very thin layer of conductive material (carbon, gold, platinum, or other metals) to ensure that the electron beam used for imaging could be dissipated from the sample surface. Coating was also used to create a layer of "electron rich" material that would serve as a source for secondary electron emission.

With the advent of environmental scanning electron microscopy (ESEM) or variable pressure scanning electron microscopes, coating of non-conductive materials is no longer a prerequisite. The atmosphere in the sample chamber itself generates positively charged ions that will dissipate any charge that accumulates on the sample surface.

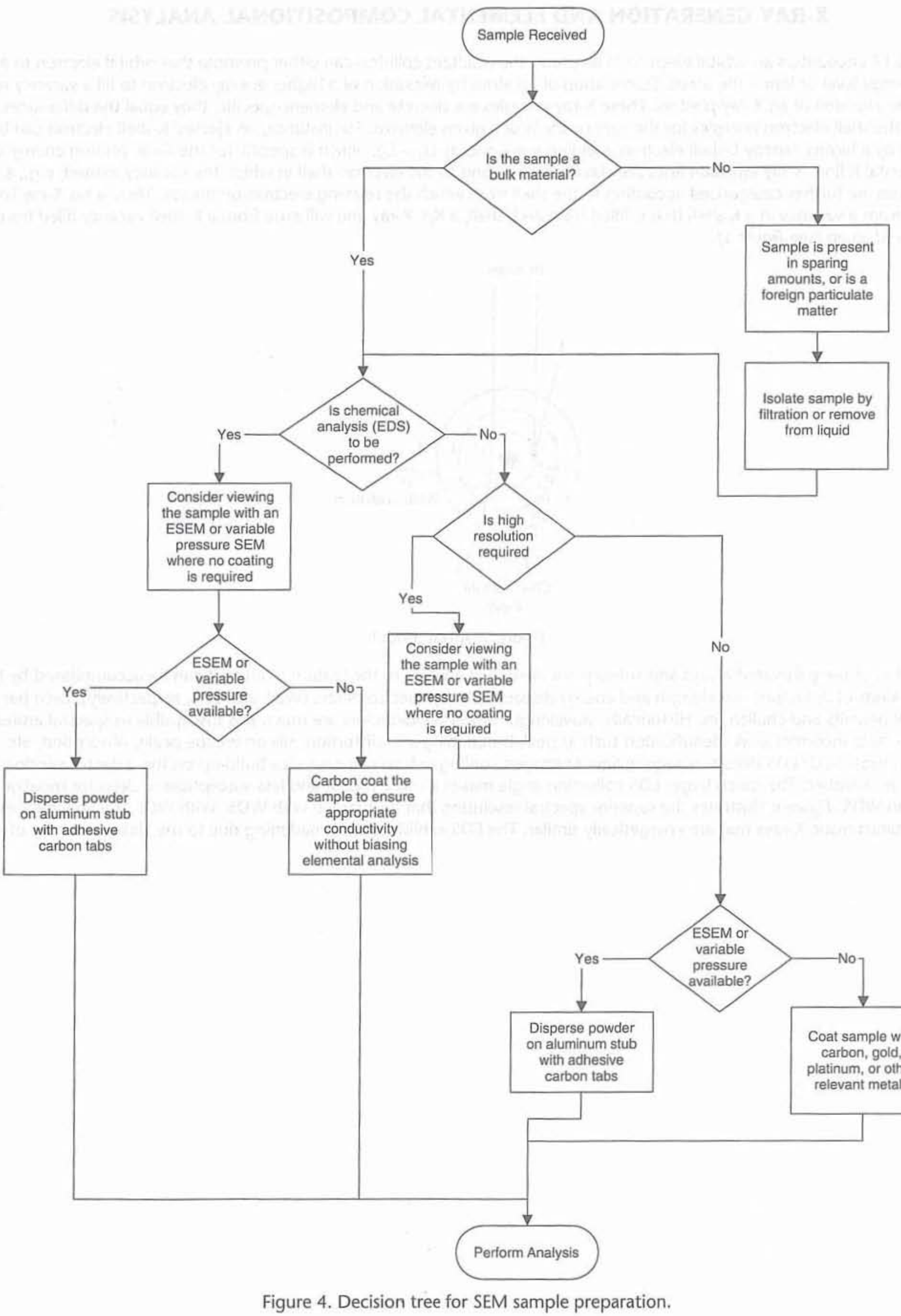


Figure 4. Decision tree for SEM sample preparation.

X-RAY GENERATION AND ELEMENTAL COMPOSITIONAL ANALYSIS

When a PE encounters an orbital electron in an atom, the resultant collision can either promote that orbital electron to a higher energy level or ionize the atom. Stabilization of an atom by relaxation of a higher energy electron to fill a vacancy results in the emission of an X-ray photon. These X-ray energies are discrete and element-specific; they equal the differences between the shell electron energies for the various shells of a given element. For instance, an ejected K-shell electron can be stabilized by a higher energy L-shell electron, yielding a net energy ($E_L - E_K$), which is specific for the X-ray photon energy of the elemental K line. X-ray emission lines are classified according to the electron shell in which the vacancy existed, e.g., K, L, M. The lines are further categorized according to the shell from which the relaxing electron originates. Thus, a Kα X-ray line will arise from a vacancy in a K-shell that is filled from an L-shell; a Kβ X-ray line will arise from a K-shell vacancy filled from an M-shell, and so on (see *Figure 5*).

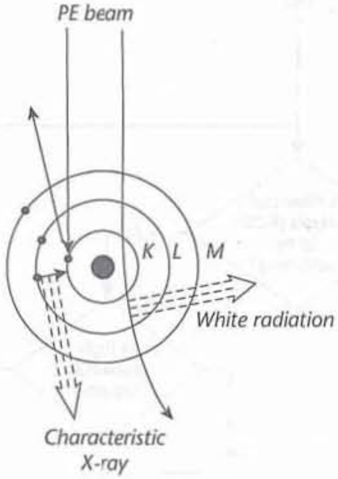


Figure 5. Atom model.

Detection of the generated X-rays and subsequent elemental analysis of the feature of interest can be accomplished by two different kinds of detectors: wavelength and energy dispersive X-ray spectrometers (WDS and EDS, respectively). Each has its individual benefits and challenges. Historically, wavelength dispersive detectors are much less susceptible to spectral artifacts that can create incorrect peak identification such as peak broadening and distortion, silicon escape peaks, absorption, etc. Additionally, most Si(Li) EDS detectors require liquid nitrogen cooling, which can cause ice build-up on the detector window in the sample chamber. The much larger EDS collection angle makes it more robust and less susceptible to detector misalignment than WDS. *Figure 6* illustrates the superior spectral resolution that is obtained with WDS. With WDS, it is much easier to detect characteristic X-rays that are energetically similar. The EDS exhibits peak broadening due to the close proximity of the peaks.

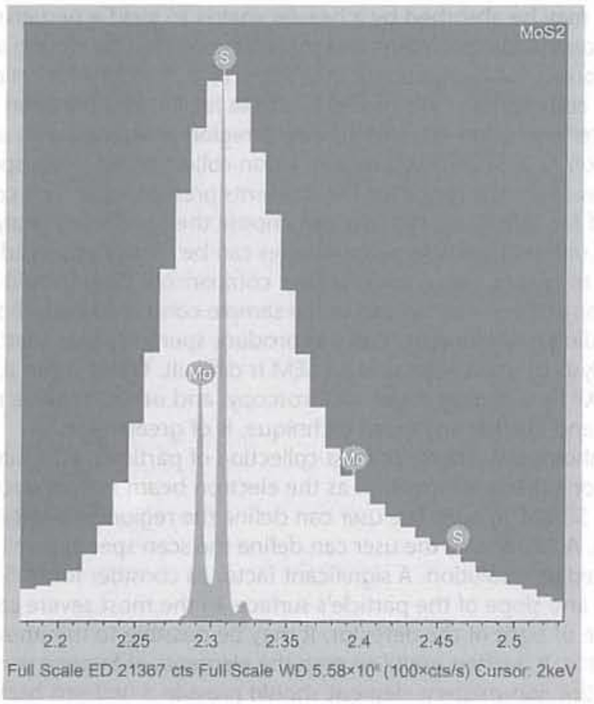


Figure 6. Example of spectral resolution obtained with wavelength-dispersive detectors where S Kα and Mo Lα can be clearly resolved by WDS (gray peaks) but are not resolved by EDS (broad yellow peak). "Used by Permission. Courtesy of Oxford Instruments."

Advantages of EDS reside in the ease of use and speed to collect the entire X-ray spectrum making it ideal for the routine identification of particles and features within a sample. The two types of detector technology most widely available for energy dispersive X-ray spectroscopy (EDS) analysis are based on lithium-drifted silicon [Si(Li)] and silicon drift technology. While Si(Li) detectors have been around for the last 40 years, silicon drift detectors (SDD) have recently emerged as the preferred type due to several key advantages including better detection of light elements ($Z \geq 4$), ability to handle high count rates, excellent energy resolution even at high count rates, and even elimination of the need for liquid nitrogen to cool the detector prior to use due to the use of Peltier cooling systems. While Si(Li) detectors were generally recommended to be run at 1,000–2,500 counts per second with an energy resolution of 129 eV at Mn-Kα, current SDD technology has superior energy resolution of 121 eV even at the high count rate of 600,000 cps. For the average user, this means that the SDD detector may drastically shorten analysis time provided that a high count rate may be obtained and with significantly better resolution for detection of light elements. In practicality, the achievable count rate is determined by the accelerating voltage and probe current, solid angle of the detector and working distance to the sample, and ability of the sample to survive the electron beam without charging or material change. Whether the user has an Si(Li) or SDD detector, it is a good rule of thumb to keep the dead time to 30% or below.

The elemental content of a specimen has a bearing on the selection of conditions for analysis. The most useful range of accelerating voltage is approximately 3–20 kV; most elements of interest can be ionized by electrons with energies in this range. The energy required in order to excite X-ray emission from a given line is termed its critical excitation potential. The critical excitation potential for a K line can be approximated by the sum of the primary line energies (Kα + Lα + Mα). Selection of an accelerating voltage equal to 1.5 times this sum is usually sufficient for semi-quantitative analysis. For example, copper has Kα at 8.05 keV + Lα at 0.93 keV = 8.98 keV: and 1.5 × 8.98 keV = 13.47 keV. Selection of 15-kV accelerating voltage yields sufficient energy to ionize the K-shell of copper atoms and generate a useful analytical signal. It is generally suggested that EDS practitioners should use the minimum accelerating voltage necessary to adequately excite the elements of interest (typically 1.5–10 times the critical excitation energy). The interaction volume increases with increasing accelerating voltage, thus better spatial resolution can be achieved by using lower accelerating voltage. Depending on the dimensions and density of the particles, a signal from the substrate may be detected. Additionally, larger interaction volume causes increased absorbance and fluorescence within the sample volume. Several accelerating voltages may be experimented with to determine the minimum accelerating voltage required. Routine use of a copper and a lighter metal standard such as aluminum allows quick verification of elemental primary line energies bracketing the 0- to 10-keV range. An elemental collection for the metal standard prior to or within the experimental protocol is conducted to verify the primary line is ±0.04 keV of reference (but may depend upon the detector manufacturer specification). This is a practical internal standard practice.

Inter-element interferences originate from many effects. High-energy X-rays emitted from heavy atoms can ionize lighter elements to produce secondary X-ray emission from the lighter species. Lower high-Z element fluorescence and higher low-Z element fluorescence can be observed, in contrast to that expected from the PE-induced signal of a pure element. Conversely, X-

ray emission from a light element may be absorbed by a heavier matrix to yield a negative bias in the light-element signal. These effects always exist in heterogeneous specimens and must be corrected for during any quantitative analysis. A common algorithm, ZAF, may be used to correct for Z-dependent interferences due to absorption and secondary X-ray emission.

Point analysis and mapping are common analysis modes available for the identification of particles by EDS regardless of detector type. Most modern user interfaces allow the user to view a region of interest with secondary electron imaging and collect a spectrum for the entire region or a defined sub-region. Upon collecting an initial spectrum, it is important the user check that it appears satisfactory in terms of energy range for the elements present, optimum count rate, and is free from artifacts such as charging. Once optimized for collection, the user can choose their preferred analysis mode to identify their sample. Point analysis is generally useful if different particle morphologies can be readily observed by SEM. The user can place a cross-hair at the center of each particle to collect a spectrum for later comparison. Care should be taken for the analysis of particles less than 5 µm since the interaction volume may spread in the sample combined with the beam/sample interaction and can excite electrons from the surrounding environment. This will produce spectral peaks that are not native to the feature of interest. For this reason, chemical analysis of small objects in an SEM is difficult. Other more surface-sensitive techniques such as X-ray photoelectron spectroscopy (XPS), scanning auger spectroscopy, and others provide more accurate spectral information. Orthogonal analysis, both within and outside any given technique, is of great value.

When there is no obvious morphological difference for a collection of particles, EDS mapping may be the best mode of analysis. In this mode, the EDS detector collects information as the electron beam rasters across the sample, thus the elemental information is in registry with the SE/BSE image. The user can define the region of interest from the entire region observable in the SE/BSE image to a sub-region. Additionally, the user can define the scan speed, dwell time/pixel, and resolution of the map to suit their needs in terms of speed or resolution. A significant factor to consider for EDS mapping is that the X-ray signal will vary based on the surface texture and slope of the particle's surface. In the most severe case, a shadow may be observed if that surface is no longer within the line of sight of the detector. It may be possible to minimize these artifacts due to tilting the sample further towards the detector. It is often useful to map the elements of interest versus an element that does not exist in the sample. The X-ray counts for the non-existent element should provide a uniform background across the entire region if the surface is level.

Examples of elemental maps overlaid on the SE image are shown in *Figure 7*. The first image (*Figure 7*, left) shows maps for barium and potassium overlaid on the corresponding SE image, thus showing that the particles represent an inhomogeneous mixture of barium/potassium precipitates (left). For the second example, EDS mapping was used with Cryo-SEM to examine a cryo-fractured silicone/water emulsion where the Si map represents silicone and the O map represents water. The internal structure of a large silicone droplet within a continuous water phase can be observed in *Figure 7* (right) where it is apparent the silicone droplet contains several internal water droplets. Many current EDS interfaces allow the user to extract a spectrum from any location within the map.

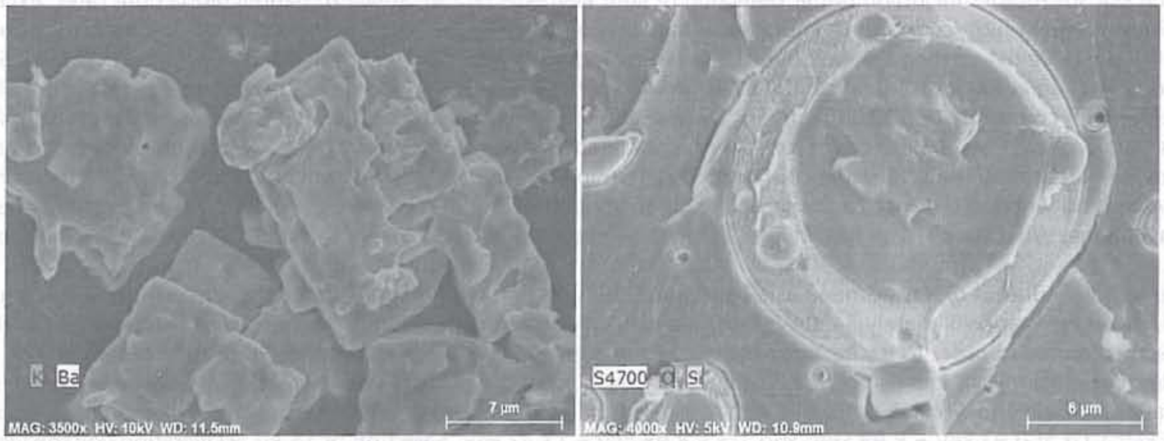


Figure 7. Left: Potassium and barium maps overlaid on the SE image of a mixed potassium/barium precipitate. Right: Oxygen and silicon maps overlaid on SE image of a vitrified silicone/water emulsion.

IMAGE OPTIMIZATION

Instrumental parameters needed to acquire a quality image can be highly dependent on sample type and sample preparation. Some important aspects with regard to the sample include the form (powders, monoliths, fibers, vitrified liquids, etc.), overall dimensions, texture, atomic composition, and inherent conductivity. Since many instrumental parameters for a modern SEM can be readily modified with the PC-based user interface with only slight re-optimization, it is highly suggested that the operator experiment with operating conditions in order to choose the best imaging conditions for the features of interest. Parameters that affect image quality can be further categorized as instrumental settings and observation settings.

Common instrumental settings that may affect image quality include accelerating voltage, emission current, working distance, SE/BSE detectors (if multiple modes are used), condenser lens current setting, and objective lens aperture diameter. Of

these, the accelerating voltage may be the most important instrumental parameter since it directly affects the penetration depth of incident electrons thereby determining the features that can be observed. In general, choosing the optimal accelerating voltage is a balance of achieving the best contrast for the features that the user is most interested in, while minimizing unwanted effects such as loss of topographic information or charging. The best approach to choosing an ideal accelerating voltage for an unknown sample is to experiment with up to 3 accelerating voltages (e.g., 1, 3, and 10 kV), then choose the best one to continue to further optimize the image. The working distance and choice of SE/BSE detector are normally chosen in combination depending on the desired resolution, depth of focus, and sample tilt. Generally, shorter working distances result in higher resolution but less depth of focus. Likewise, based on the position the SE detectors are placed in the column, a tradeoff exists for spatial resolution versus surface topography, where upper detectors generally detect a high resolution SE signal and lower detectors show more surface topography. Lastly, the condenser lens setting and objective lens aperture diameter can be optimized to provide the ideal specimen current, resolution, and depth of focus required for a specific sample type.

Upon choosing an initial set of instrumental conditions as mentioned above, basic image observation can be optimized in the following manner:

(1) Select a low magnification (1000–3000×) initially
(2) Course adjust image brightness
(3) Course adjust focus
(4) Select an easy-to-visualize area of the specimen
(5) Check/adjust to optimize the electromagnetic alignment of the electron optics (beam, aperture, stigmation)
(6) Select a feature of interest at higher magnification
(7) Adjust focus and astigmatism
(8) Adjust image brightness and contrast
(9) Collect image.

Image collection can be accomplished using different resolutions (1280 × 960 is most common) and capture speeds using either slow scan collection or frame integration. In general, slow scan collection is used to collect high-resolution images whereas frame integration is used for specimen types that have charging or stability issues. A collection using a higher capture resolution, slower scan speed, or additional frames may improve the image resolution, however the user must take caution of the increased likelihood of specimen drift over this period which would negate any benefit.

FORENSIC ANALYSIS OF PARTICLES

Shape Analysis

Because of the three-dimensional nature of images obtained via scanning electron microscopy, particle shape provides a great deal of information about the composition and source of the particle without obtaining a chemical analysis via EDS.

The most commonly identified particles found in pharmaceutical compounds generally fall into several distinct categories. Note that particles in parenteral products arise from three general sources: (a) extrinsic or outside of the product, as truly foreign solids; (b) intrinsic or associated with the product, its package or manufacture; (c) inherent particles expected to compose the formulation, such as proteins in biotherapeutic products (see *Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨787⟩), and may be encountered when probing those formulae. Some examples of extrinsic particles are the following:

- Corrosion products from one or more equipment sources, and may occur from formulation interaction
- Materials from the manufacturing environment such as cellulose (from paper bags), extraneous fibers (clothing, filters, etc.)
- Inhomogeneities in the product, specifically incomplete mixing (for solid oral dosage products) that can create cosmetic or elegance issues with tablets
- Environmentally-sourced biological materials
- Material that has been exposed to extreme heat that might char. The causes for this can vary from paper pyrolized during depyrogenation to material sticking to components that are exposed to friction (powder milling, compaction, etc.). If residue unintentionally builds up in these areas, the exposure to friction can decompose the material, making it appear burnt.

Figure 8 shows representative images of some of the particulate matter mentioned above that the user might encounter in a forensic investigation.

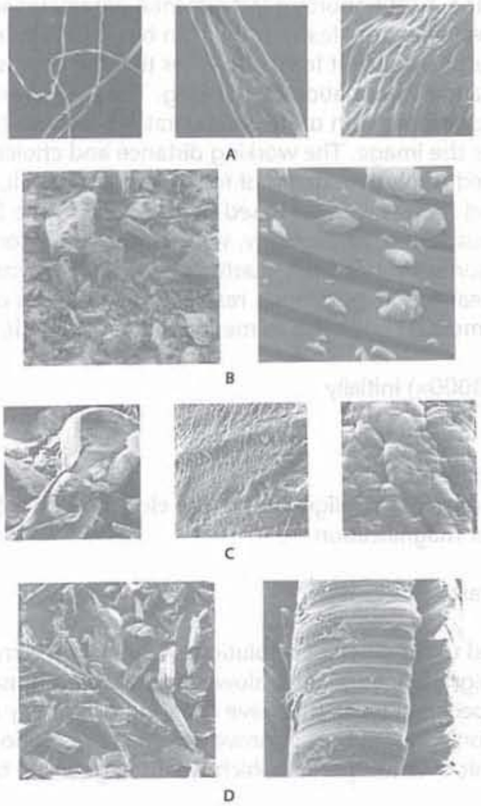


Figure 8. Scanning electron micrographs of commonly found particles in pharmaceutical manufacturing areas: A. cotton fibers, B. glass particles, C. Teflon shavings, D. aluminum shavings. (All of the images provided courtesy of McCrone Atlas (www.mccroneatlas.com.)

Interpretation of a particle's origin from characteristics observed by SEM analysis depends on the particle features; for instance, any particle with sharp corners and well-defined edges is made from a material that is not soluble in the product liquid. If it is transparent to the beam, then the user could conclude it consists of very low Z elements. Crystalline particles exhibit obvious symmetry and growth patterns. Slow growth is characterized by large crystals and fast growth by small, often aggregated crystal masses.

Particle morphology and surface characteristics may be helpful in sourcing unknowns. Aspect ratio, or the ratio of the length of a particle divided by its width, indicates fibrous materials. Analysis by optical microscopy often serves as a more effective tool to analyze fibers by determining diagnostic optical properties (refractive indices, birefringence, elongation, etc.) using plane and crossed polarized light that can distinguish among various possible sources. With a scanning electron microscope, however, elemental analysis can determine (a) if the fiber is inorganic or organic and (b) the presence of surface additives. Scanning electron microscopy also offers the added benefit of being able to image at much higher magnification to reveal morphological structures of biological origin or features indicative of commercial manufacture.

As mentioned previously, different kinds of electrons are emitted when an electron beam interacts with a solid surface. The two most useful types of electrons are secondary and back-scattered. By choosing the appropriate detector or instrument set up, the image can be biased towards one or the other. Back-scattered electrons can provide useful information regarding the chemical composition of the feature in question. If the instrument is set up to use BSE to form the image, elements possessing higher atomic mass will appear brighter than those having a lower atomic mass. By selecting this imaging mode, organic particles are easily distinguished from metallic debris, for example. For instance, at low kV, glass or plastic may appear as solid objects, whereas at higher kV they may appear somewhat transparent. Further, appearance at low kV on a tungsten filament SEM may mean something completely different than that at low kV on an FE SEM or environmental SEM. Different materials require different operating conditions to suitably image using SE, to prevent charging and to improve contrast. Typically, use low kV (3 kV or less using a semi-in-lens or in-lens FE SEM) for non-conductive materials such as glass and plastic. If the material is extremely thin (<100 nm), the material can be visualized in transmission mode (STEM) as well as SE mode.

GENERAL REFERENCES

1. Goldstein, J., et al., *Scanning Electron Microscopy and X-ray Microanalysis*. Third ed. 2003, New York: Springer.

2. Echlin, P., *Handbook of Sample Preparation for Scanning Electron Microscopy and X-ray Microanalysis*. 2010, New York: Springer. 330.
3. Reimer, L., *Scanning Electron Microscopy: Physics of Image Formation and Microanalysis*. Springer Series in Optical Sciences, ed. H.K.V. Lotsch. Vol. 45. 1998, New York: Springer.

⟨1184⟩ SENSITIZATION TESTING

INTRODUCTION

This chapter considers sensitization and hypersensitization in the context of medical devices and implants, and describes methodologies for testing such articles for their potential to cause sensitization.

There are four types of hypersensitization reactions according to the Gell and Coombs classification system. Type I reactions involve the fixation of IgE to mast cells that subsequently release pharmacologically active substances, such as histamine. Type II reactions are the result of IgG and/or IgM binding to target cells, followed by complement fixation and cell lysis. Type III reactions are caused by the presence of antigen-antibody complexes that cause physical damage such as kidney damage due to glomerular blockage. Type IV reactions are cell-mediated (involve the action of T cells and their interaction with the human lymphocyte antigens). Type IV reactions are also called delayed-type hypersensitivity reactions. *Table 1* below summarizes the types of reactions, the mediators of the reactions, and examples of representative diseases.

Table 1. The Four Types of Hypersensitization Reactions*, Mediators, and Disease Examples

Reaction Class	Mediators	Disease Examples
Type I	IgE molecules bound to mast cells interact with antigen to release pharmacologically active substances	Hay fever, bronchial asthma, other atopic reactions
Type II	IgM and/or IgM molecules interact with target cells, fix complement, cell lysis	Various drug allergies, erythroblastosis fetalis, hemolytic anemia, thrombocytopenia
Type III	Antigen-antibody complexes, complement	Arthus reaction, serum sickness, allergic glomerulonephritis
Type IV	T lymphocytes, antigen, monocytes, macrophages	Contact dermatitis

* According to Gell and Coombs classification scheme

A multi-step process, delineated in chapter *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* ⟨1031⟩ is followed in determining which, if any, toxicological tests need to be performed on a given article. In some cases, sufficient evidence to satisfy toxicology requirements may be available from previously marketed articles (See *Figure 1* in chapter ⟨1031⟩). Important factors addressed in *Figure 1* (chapter ⟨1031⟩) include the type and extent of contact with the body, the chemical composition, the manufacturing process, the sterilization process, and, as mentioned above, similarity to previously marketed articles.

If further toxicological testing is necessary, the classification of medical devices provided in *Table 2* from general information chapter ⟨1031⟩ is important, because the degree and extent of toxicological testing that is required is strongly influenced by the nature and duration of the bodily contact with the article. The classification derived from *Table 2* in chapter ⟨1031⟩, coupled with the length of exposure to the article, is used in *Tables 3–5* of chapter ⟨1031⟩ to determine which toxicological tests need to be performed. *Table 2* below presents information extracted from *Tables 3–5* of chapter ⟨1031⟩ and indicates those circumstances for which sensitization testing should be considered.

Table 2. Articles For Which Sensitization Testing Should Be Considered Based on Article Category and Length of Exposure

Device Category	Body Contact	Contact Duration
Surface devices	Skin	A[a], B[b], C[c]
	Mucosal membrane	A, B, C
	Breached or compromised surfaces	A, B, C
External communicating devices	Blood path, indirect	A, B, C
	Tissue, bone, or dentin communicating	A, B, C
	Circulating blood	A, B, C
Implant devices	Tissue or bone	A, B, C
	Blood	A, B, C

[a] A: limited (less than 24 hours)
[b] B: prolonged (24 hours to 30 days)
[c] C: permanent (more than 30 days)

There are nine test methodologies reviewed in this chapter. *Table 3* lists the methods and the species with which they are performed.

Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test

Test	Species Used in Test
Magnusson & Kligman Maximization	Guinea pig
Standard Buehler	Guinea pig
Open Epicutaneous	Guinea pig
Freund's Complete Adjuvant	Guinea pig
Optimization	Guinea pig
Split Adjuvant	Guinea pig
Local Lymph Node Assay	Mouse
Mouse Ear Swelling	Mouse
Vitamin A Enhancement	Mouse

Given the preponderance of testing performed with either the *Magnusson & Kligman Guinea Pig Maximization Test* (GPMT) or *Buehler Tests* (BT), those tests will be reviewed in detail in this chapter. A brief summary of the remaining tests is provided as alternatives to the more frequently used procedures.

Each test should be periodically validated in the performing laboratory using positive controls such as hexyl cinnamic aldehyde, mercaptobenzothiazole, or benzocaine (positive controls recommended by the Organization for Economic Cooperation and Development [OECD]).

MAGNUSSON & KLIGMAN GUINEA PIG MAXIMIZATION TEST (GPMT)

Animals

Either male and female albino guinea pigs or both may be used. All animals should be in good health and weigh between 300 g and 500 g at the start of the experiment. The females should not be pregnant, nor should they have borne young previously. Prior to use, it is essential to acclimatize the animals to the laboratory conditions for at least 5 days. All animals should be handled in accordance with the guidelines in the appropriate regulatory requirements established for the humane treatment of animals. At least 10 test animals and 5 control animals should be used. To obtain sufficient analytical power (i.e., to detect weak sensitizers) it may be necessary to use 20 test animals and 10 control animals. Additional animals may be required to establish the proper doses to administer (see *Determination of Test Article Concentration*).

Housing and Feeding

The animal room should be held at 20 ± 3°, at 30% to 70% relative humidity, with 12 hours of light and dark. Animals may be housed individually or in group housing. Standard laboratory diets may be used (those satisfactory for guinea pigs ensure an adequate amount of ascorbic acid). Drinking water should be available ad libitum.

Animal Pretest Preparation

Animals should be randomized via a validated randomization method. For example, such methods may utilize random number tables or computer-generated random numbers. Sites on the animals intended for test article application (intrascapular region) should have the hair removed in a manner that does not abrade the skin. This may be accomplished via clipping, shaving, or with chemical depilatories. The chemical depilatory must not elicit irritation of its own. General observations of the animals prior to use in the test should be recorded, including any indication of ill health (do not use such animals in tests), and body weights.

Test Article Preparation[1]

The use of this test requires that the test article can be injected intradermally. When the test article is not suitable for direct administration, extracts should be prepared according to the procedure provided in general chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩.

[1] For further information on sample preparation, see ANSI/AAMI/ISO/CEN Standard 10993–12—1996: Biological Evaluation of Medical Devices—Part 12: Sample Preparation and Reference Materials.

Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of a GPMT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be injected intradermally (0.1 mL per site), using the solvent that will be employed in the *Test Procedure.* The concentration that causes only mild to moderate irritation (no extensive skin destruction, with no evidence of overt systemic toxicity to the animals) should be used in the *Intradermal Injection Induction Phase* of the *Test Procedure.*

Using two or more animals, apply via occlusive dressings and patches, a range of concentrations of test article or extracts of the article. Remove the dressings/patches after 24 hours, and examine the sites for erythema. Choose the concentration that causes only slight erythema for the *Topical Application Induction Phase* of the *Test Procedure.* Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure.* If the irritation threshold is not reached, then select the highest possible concentration for the *Topical Application Induction Phase* and *Challenge Phase* of the *Test Procedure.*

Test Procedure

INTRADERMAL INJECTION INDUCTION PHASE

This phase requires three pairs of injections administered intradermally, with the test and control injection of each pair on opposite sides intrascapularly. Each injection should contain 0.1 mL, with injection pairs 1 and 2 administered nearer to the head, and injection pair 3 administered slightly farther towards the tail. The pairs are nominally within an area of 8 cm². The pairs of injections consist of the following:

Injection pair 1:	A 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA), an oil–water emulsion containing mycobacteria, and the appropriate solvent/vehicle (see *Biological Reactivity Tests, In Vivo* ⟨88⟩). Control animals receive a mixture of FCA and physiological saline (1:1).
Injection pair 2:	The *Test Article Preparation* in the concentration as specified in *Determination of Test Article Concentration,* using the appropriate solvent/vehicle. Control animals receive only the solvent/vehicle.
Injection pair 3:	The *Test Article Preparation* in the concentration as specified in *Determination of Test Article Concentration* in a 1:1 (v/v) mixture with FCA. Control animals receive an injection of a 1:1 (v/v) mixture of FCA and solvent/vehicle.

TOPICAL APPLICATION INDUCTION PHASE

Seven days (±1 day) after completion of the *Intradermal Injection Induction Phase,* administer the test sample by topical application to the intrascapular region of each animal. For both test and control animals, if the *Test Article Preparation* does not cause skin irritation, apply 10% sodium lauryl sulfate in petrolatum approximately 24 hours before the start of the *Topical Application Induction Phase* to induce a local irritation.

Test animals should have 2- × 4-cm pieces of filter paper or absorbent gauze fully loaded with the *Test Article Preparation* (prepared within 24 hours of use) using the concentration selected in *Determination of Test Article Concentration* applied to each injection site. The filter paper or absorbent gauze should be secured to the animals using occlusive dressings. Control animals receive the same treatment, except that the appropriate solvent/vehicle is used instead of the test article.

Remove the dressings and patches approximately 48 hours after application.

CHALLENGE PHASE

This phase should occur 14 ± 1 days after the *Topical Application Induction Phase.* Hair should be removed from the test application sites. Filter paper patches or chambers are soaked with a freshly prepared *Test Article Preparation* in the concentration specified in *Determination of Test Article Concentration.* This is done for all test and control animals. The patches or chambers are secured with an occlusive dressing and removed after 24 ± 2 hours.

Observations

At approximately 24, 48, and 72 hours after removal of the challenge patches, the application sites should be examined for signs of reactions. Of particular importance are instances where the reaction of the test animals exceeds that of the control animals. All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. A true edematous reaction will blanch under gentle pressure. The longer the period of blanching, the greater the severity of edema.

Interpretation

There is more than one way of evaluating and grading the results from GPMT. *Tables 4*, *5*, and *6* list details for three such grading systems. Grades of 1 or higher in the test animals, with grades of less than 1 in control animals, are indicative of sensitization. If control animals display grade 1 reactivity, and if the test animals display reactivity above the greatest reactivity seen in the control animals, sensitization due to the test article is again suspected. The percentages in *Table 4* need to be revised if there are only 10 test animals (i.e., the categories would be 0, <10%, 10%–30%, 31%–60%, 61%–80%, and 81%–100%.) If there are 20 test animals, then multiples of 5% are appropriate.

Table 4. Classification Based on Percent of Responsive Test Animals

% of Positives in Test Group	Assigned Grade Class	
0	—	Nonsensitizer
<8	1	Weak
8–28	2	Mild
29–64	3	Moderate
65–80	4	Strong
81–100	5	Extreme

Table 5. Classification Based on Erythema and Edema Formation

Erythema and Eschar	Grade
No erythema	0
Slight or equivocal erythema	<1
Well-defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Edema	
No edema	0
Slight or equivocal edema	<1
Well-defined edema	2
Moderate edema	3
Severe edema	4

Table 6. Classification Based on Erythema Formation Alone

Erythema formation	Grade
No erythema	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

The results should be submitted for statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

Rechallenge

The extent of any response in the negative control group, under experimental conditions, shows the irritation potential of the *Test Article Preparation*. In this case, test and control animals should be rechallenged 1 week later on the untreated side of the animal, with a reduced concentration of the *Test Article Preparation*. A sensitized guinea pig will react to some degree to both challenges. A weak reaction occurring at a single time point in only one challenge should cast strong doubt as to whether that guinea pig is truly sensitized.[2]

[2] Basketter D.A. Guinea pig predictive tests for contact hypersensitivity. In Immunotoxicology and Immunopharmacology, 2nd ed.; Dean, J.H, Luster, M.I., Munson, A.E., Kimber, I., Eds; Raven Press, Ltd: New York, 1994; pp 693–702.

STANDARD BUEHLER TESTS (SBT)

Animals

See *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

Housing and Feeding

See *Housing and Feeding* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

Animal Pretest Preparation

See *Animal Pretest Preparation* under *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).* The fur of the guinea pig may be removed from one flank by clipping.

Test Article Preparation

See *Test Article Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of an SBT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be applied using patches (for example, four 4 cm^2 absorbent pads) or chambers. The patches should be held in place using tape (if necessary) and occlusive dressings. The patches should be removed after approximately 6 hours, and any residues of the test chemical are removed from the test site. Observations are made at that time, and at 24 and 48 hours.

The concentration that causes only mild to moderate irritation (slight erythema, with no evidence of overt toxicity to the animals) and can be applied repeatedly to the same site should be used in the *Induction Phase* of the *Test Procedure.* Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure.*

Test Procedure

INDUCTION PHASE

Apply 0.4 mL of the *Test Article Preparation* in an appropriate solvent/vehicle at the dose identified in *Determination of Test Article Concentration.* Use patches similar to those used in *Determination of Test Article Concentration.* The patches should be applied to one flank (hair clipped off) and held in place occlusively for 6 hours. The animals may need to be restrained to ensure occlusion. Patches and any visible residues should be removed after 6 hours. Control animals also receive patches, but these contain only the appropriate solvent/vehicle. This process should be repeated three times a week for both test and control animals on the same site for three consecutive weeks (weekly intervals are used in the modified Buehler Test).

CHALLENGE PHASE

This phase should be carried out 14 days after the last application of the *Induction Phase.* Clip the hair off the previously untested flank of each animal 24 hours before the challenge application. As in the *Induction Phase,* apply patches containing the test article (concentration specified in *Determination of Test Article Concentration*) or solvent/vehicle alone to the untested areas of the test and control animals. To obtain well-defined edges at the application sites, commercial chambers with a lipped edge are preferred. Secure the patches with occlusive dressings, and keep them in place for 6 hours. Remove all patches after 6 hours.

OBSERVATIONS

At 22 ± 2 hours after removal of the patches, the application sites should have the animal's fur removed via clipping or depilation. After approximately 2 more hours, grade the sites (*Tables 4, 5* or *6* may be employed). All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. Repeat the grading once again after 24 to 48 hours more have elapsed. The response of the test group versus the control group can be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

INTERPRETATION

The results should be submitted for a statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant.

See *Interpretation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

RECHALLENGE

See Rechallenge in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT).*

OTHER SENSITIZATION TEST PROCEDURES

The *Magnusson & Kligman Guinea Pig Maximization Test* and the *Standard Buehler Tests* are the most frequently performed sensitization tests. However, there are a number of other methods that may be useful in the assessment of the potential for sensitization. Some may be applicable to both solid test articles and extracts, some only to extracts.

Where the use of guinea pigs is called for in the following tests, the animals and their housing should meet the requirements as specified for *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test.* The fur of the guinea pig should be removed from test sites as indicated for *Animal Pretest Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test.*

Draize Test

This was the first predictive test accepted by the regulatory agencies, and is still in use. The test uses guinea pigs and the test article is administered via intradermal injections.

TEST ARTICLE PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material would need to be made. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the preparation procedure.

INDUCTION PHASE

One flank of each of 20 guinea pigs is shaved, then 0.05 mL of a 0.1% solution of test article is injected into the anterior flank. The next day, and then every other day thereafter up to day 20, 0.1 mL of the test article is injected into a new site on the same flank.

CHALLENGE PHASE

This phase begins 2 weeks after the final injection of the *Induction Phase.* The untreated flank is shaved, then 0.05 mL of test article is injected into each of the 20 guinea pigs. Twenty previously untreated animals serve as the controls, and receive injections of the test article as well.

OBSERVATIONS

The test sites of all control and test animals are evaluated for erythema at 24 and 48 hours after the challenge injections. The degree of reaction in test animals is compared to the reaction in control animals. A larger and/or more intense response by the test animals versus the control animals is indicative of sensitization.

Open Epicutaneous Test

This test uses guinea pigs. The goal is to determine the dose required to induce sensitization by simulating human usage via topical application of the test article.

TEST MATERIAL PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material need to be made. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the preparation procedure.

PRELIMINARY TESTING

A series of concentrations of test article is applied to 2 cm^2 areas of skin on the anterior flank of 6 to 8 guinea pigs (0.025 mL per application). The test sites should be examined for erythema 24 hours after test article administration. The highest concentration that does not cause irritation (maximum nonirritant concentration) and the lowest concentration causing erythema in approximately 25% of the animals (minimum irritant concentration) are determined.

INDUCTION PHASE

The test article (or control vehicle) is applied to 8 cm^2 areas of the flank skin of 6 to 8 guinea pigs daily for 3 weeks, or five times a week for 4 weeks. The amount per application is 0.01 mL. A set of increasing concentrations is again employed, ranging from the minimum irritant concentration using a stepwise progression. The test article should be applied to the same sites each time, unless irritation develops, in which case a new site on the same flank should be used. Control animals receive the same series of treatments using the vehicle instead of the test article.

CHALLENGE PHASE

Each animal is challenged on the untreated flank 24 to 72 hours after the last *Induction Phase* treatment using 0.025 mL applied to 2 cm^2 areas. A set of increasing concentrations is used, from minimum irritant concentration to the maximum nonirritant concentration, and five lower concentrations are also used.

OBSERVATIONS

The test sites are evaluated at 24, 48, and 72 hours post-treatment. The maximum concentration that does not cause irritation in the control group is determined. Animals from the test groups that develop inflammatory responses at concentrations lower than the maximum nonirritating concentration in the controls should be considered to be sensitized.

Freund's Complete Adjuvant Test

This test is based upon the use of intradermal injections using the test article in a mixture of Freund's complete adjuvant and distilled water (50:50).

TEST MATERIAL PREPARATION

Because this test uses intradermal injections, extracts of the test material need to be made in order to use this procedure. See *Biological Reactivity Tests, In Vivo* 〈88〉 for information on the extraction procedure.

PRELIMINARY TESTING

The minimum irritating and the maximum nonirritating concentrations are determined in the same manner as for *Preliminary Testing* in the *Open Epicutaneous Test*.

INDUCTION PHASE

The test area consists of six 2 cm^2 areas across the shoulders of the guinea pigs. Two groups of 10 to 20 guinea pigs each should be used. The test group animals are injected intradermally with 0.1 mL of a 5% solution of the test article extract in FCA/water. Control animals receive injections with FCA/water without the test article. These injections are repeated every 4 days until a total of three injections have been given.

CHALLENGE PHASE

This phase should begin 2 weeks after the last injection of the *Induction Phase*. Topical applications of 0.025 mL of test article at the minimum irritating and the maximum nonirritating concentrations, plus two lower concentrations, are administered to 2 cm^2 areas of the shaved flank. The test sites should remain uncovered.

General Chapters

OBSERVATIONS

The test sites are examined for the presence of erythema 24, 48 and 72 hours after the topical applications. The minimum nonirritating concentration in the control animals should be determined. Those test animals that display erythema at concentrations lower than the minimum nonirritating concentration in the control animals should be considered to be sensitized.

Optimization Test

This test has some similarities to the older *Draize Test.* Unlike the *Draize Test,* however, this test uses both intradermal and topical treatments, and includes adjuvant for some induction injections.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction procedure.

INDUCTION PHASE

Twenty test and 20 control guinea pigs are used. A total of 10 intradermal injections should be given to each animal. Test animals receive 0.1 mL of a mixture of 0.1% test article and 0.9% saline (50:50) on day 1, with one injection into a shaved flank, and another into a portion of shaved dorsal skin. Two and 4 days later, one intradermal injection of the test article in saline is given to eight new dorsal sites. Every other day during weeks 2 and 3, the test article is injected intradermally into 10 sites over the shoulders in a 50:50 mixture of saline and FCA. The same sequence of injections is given to the 20 control animals, except that no test article is included with the saline or saline/FCA injections.

CHALLENGE PHASE

Thirty-five days after the first injection, the animals are challenged topically with 0.1 mL of the 0.1% solution of test article in saline (for test animals). The control animals receive saline injections only. At 45 days after the first injection, a second topical challenge is given. A nonirritating concentration of test article (0.05 mL) is applied topically to a 1 cm^2 area of untreated skin. This site should then be covered with a 2 cm^2 piece of filter paper, after which an occlusive dressing should be applied. The patch should be removed after 24 hours.

OBSERVATIONS

Twenty-four hours after each injection during week 1, the thickness of a fold of skin over the injection sites for each animal should be measured using a caliper (mm), and the two largest cross-diameters of each erythematous reaction should be recorded (mm). The reaction volumes are calculated by multiplying the fold thickness by the products of the two cross-diameters (expressed as µL). The mean reaction (+1 SD) volume during week 1 should be calculated for each animal.

Challenge reaction volumes are calculated for each animal following the injections at day 35. If an animal develops a challenge reaction volume greater than its mean reaction volume + 1 SD, it should be considered sensitized.

Following the patch testing challenge, the test sites are evaluated for erythema and edema. Evaluations should be made using *Table 5.*

The number of positive animals should be compared statistically with the pseudopositive control animals. This should be done for both intradermal injection results and patch testing results. The Fisher exact test may be used.

The results from the intradermal injections and the patch testing, following separate statistical analysis, may be combined and evaluated using *Table 7* in order to classify a test article as a strong, moderate, or weak sensitizer; or not a sensitizer.

Table 7. Classification Scheme for Test Articles Based on the Optimization Test

Intradermal % of Positive Animals	Patch Test % of Positive Animals	Classification
S*, > 75	and/or S, > 50	Strong sensitizer
S, 50–75	and/or S, 30–50	Moderate sensitizer
S, 30–50	N.S.*, 0–30	Weak sensitizer
N.S., 0–30	N.S., 0	Not a sensitizer

* S = significant; N.S. = not significant

Split Adjuvant Test

This test makes use of both FCA and skin damage. The test article is applied topically.

TEST MATERIAL PREPARATION

Because this test employs topical test article applications, the article can be either in solid or liquid form. If extracts are to be made, see chapter *Biological Reactivity Tests, In Vivo* 〈88〉 for extraction procedures.

INDUCTION PHASE

Ten to 20 guinea pigs are used for both test and control groups. An area of back skin immediately behind the scapulas should be shaved to the extent that the skin becomes glistening. The shaved areas should then be treated with dry ice for 5 to 10 seconds. A dressing made of loose mesh gauze with stretch adhesive and a 2- × 2-cm opening should be placed over the treated area, then secured with adhesive tape. The test article (0.2 mL of viscous materials, 0.1 mL of liquids, or solid material) is placed within the opening in the dressing on top of the treated skin. Two layers of #2 filter paper should be placed over the test article, then backed by occlusive tape. Then the filter paper/occlusive backed material should be secured to the surrounding dressing with adhesive tape. After 2 days have passed, the filter paper should be lifted from the test sites, and the test article reapplied on the same site. The filter paper and backing should be secured once again. After 2 more days, the filter paper should be lifted and two injections of 0.075 mL of FCA should be administered into the edges of the test site. Then the test material is once again applied, and the filter paper/backing resecured. The test article should be reapplied once more on day 7 and the filter paper/backing resealed. On day 9, the filter paper and all associated dressing material should be removed.

CHALLENGE PHASE

On day 22 following the induction treatment, 0.5 mL of test material (or the solid article) should be applied to a 2-× 2-cm area of shaved midback. The test sites should be covered by filter paper and backed by adhesive tape. This should be held in place with an elastic bandage secured with adhesive tape. Control animals receive the same challenge phase treatment. The preparation should be removed after 24 hours.

OBSERVATIONS

Twenty-four, 48, and 72 hours after the removal of the challenge phase preparation, the test sites should be evaluated for erythema and edema. The grading scheme of *Table 5* could be employed.

Mouse Ear Swelling Test

There are a number of potential advantages in using mice versus guinea pigs for sensitization methods. The classic guinea pig tests tend to be costly and require a long time to complete. Moreover, with the dependence upon relatively subjective scoring based on edema and erythema, methodological robustness, and ruggedness may be questionable. This test uses mice and employs both topical exposures and injections.

ANIMALS

Female, 6- to 8-week old CF-1, Balb/c, or Swiss mice should be used. They may be group housed in direct bedding cages. Acclimatization should be for at least 5 to 7 days. Food (appropriate mouse feed) and water should be available ad libitum. No animals with damaged pinnae should be used in the study. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* 〈88〉 for information on the extraction procedure.

PRELIMINARY TESTING

The minimally irritating and maximally nonirritating concentrations of test article for this procedure should be determined. This is done by using four groups of two mice and examining the effects of at least four concentrations of test article.

INDUCTION PHASE

The abdomens of the animals should be shaved, then tape-stripped using a surgical adhesive tape until the test area is glistening. A single injection of 0.05 mL of FCA is subdivided into two injection sites administered intradermally within the shaved/stripped area, but along the borders. After the adjuvant injections, 100 µL of test article (using the minimally irritating concentration) or vehicle (controls) is applied to the center of the shaved test areas. After the test areas dry, the mice should be returned to their cages. The tape stripping and application of test article (but not FCA) is repeated each day for the next 3 days.

CHALLENGE PHASE

This phase should occur 7 days after the final topical induction application. The test article (highest nonirritating concentration) should be applied topically (20 µL) to one ear, while the opposite ear receives 10 µL of vehicle alone. This should be done for both test and control animals.

OBSERVATIONS

The thickness of both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). A sensitized animal is one in which the test article-treated ear is at least 20% thicker than its opposite ear. For the test to be valid, the test article-treated ears of control animals should not be more than 10% thicker than the opposite ears. If the control animal ears do not meet the requirements, the test should be repeated using lower concentrations.

Local Lymph Node Test

This test is based on the observation that exposure of the mice to sensitizers can cause hyperplasia of T cells within the auricular lymph nodes of mice. The method combines both in vivo and in vitro phases, and requires the use of radioisotopes. An unusual aspect of this test is that no challenge phase is required.

ANIMALS

Four groups of four mice at least, male or female CBA/ca mice (only one sex in a given test) between the ages of 8 to 12 weeks should be used.

TEST MATERIAL PREPARATION

Although in theory one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* 〈88〉 for information on the extraction procedure.

PRELIMINARY TESTING

A nontoxic concentration of test article should be used. If not already established, a preliminary test for overt toxicity may be required to establish a suitable dose.

INDUCTION PHASE

Twenty-five µL of the appropriate test article concentration, or vehicle (controls), should be applied to the dorsal surface of each pinna for 3 consecutive days. Five days after the first treatment, the animals should be injected, via the tail vein, with 2.5 mL of phosphate buffered saline containing 20 µCi of ^{3}H-methyl thymidine. Five hours after the isotopic injection, the animals should be euthanized. The draining auricular lymph nodes should be removed from each animal of each test and control group. The nodes from all animals within a given group should be combined, such that a single cell suspension can be made from each group of animals. The cell suspension can be made by passing the nodes through a 200-mesh stainless steel gauze using a syringe plunger. The cells should then be centrifuged at 190 × g for 10 minutes, resuspended in 3 mL of 5% trichloroacetic acid (TCA), and held overnight at 4°.

The resulting precipitate should be recovered by centrifugation, and the pelleted precipitate should be resuspended in 1 mL of 5% TCA. The suspension should then be placed in scintillation vials with 10 mL of scintillation fluid, and the disintegrations/minute (dpm) counted with a β-counter.

OBSERVATIONS

The ratio of dpm for each test group should be compared to the dpm for the control group. If the ratio equals or exceeds 3 for any test group, the concentration of test article used with that group may be considered to be sensitizing.

Vitamin A Enhancement Test

This test is similar to the *Mouse Ear Swelling Test* in that test articles are applied topically to the abdomen, with a challenge application to the ears, followed by measurements of ear thickness. A principal difference is the use of mouse feed supplemented with vitamin A acetate. The purpose of the supplementation is to increase the reactivity of the immune system, thereby increasing the potential sensitization reaction.

ANIMALS

Male, 3- to 4-week old Balb/c mice should be maintained on a diet supplemented with vitamin A acetate. The diet may be prepared by mixing each kg of feed with 0.477 g of gelatinized vitamin A acetate. The feed mixture should be used within 3 weeks of preparation. Mice intended for use in sensitization studies should have been on the supplemented diet for at least 4 weeks. The mice at the time of the sensitization study should therefore be between 7 and 10 weeks old. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

Although, in theory, one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* ⟨88⟩ for information on the extraction.

PRELIMINARY TESTING

The maximally nonirritating dose and minimally irritating concentrations should be determined using separate groups of animals. This could be done as described for *Preliminary Testing* in the *Mouse Ear Swelling Test*.

INDUCTION PHASE

The fur of the abdomen and thorax of 10 mice per group should be shaved. Then 100 µL of test article (at the minimally irritating concentration) should be applied to the test areas on days 0, 2, 4, 7, and 11. Control animals receive 100 µL of vehicle alone on the same schedule.

CHALLENGE PHASE

This phase should occur 4 days after the final application of the *Induction Phase*. Twenty-five µL of test article (at the maximally nonirritating concentration) should be applied to each ear of each animal in the test and control groups.

OBSERVATIONS

Ear thickness for both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). The percent increase in ear thickness should be calculated for each ear by subtracting the pretreatment measurement from the post-treatment measurement, dividing the result by the pretreatment measurement, then multiplying by 100. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test could be used for the comparison.)

The results of individual animals should also be calculated. If an increase in ear thickness for an animal from the test group is at least 50% greater than the largest increase of a control animal, that is indicative of sensitization. As an overall evaluation, should the results of the study provide a significant result of the statistical test at $p < 0.01$ for the control versus test group comparisons, or if at least two test animals have ear thickness increases in excess of 50% of the maximum control thickness changes and the group comparison showed a $p < 0.05$, sensitization is indicated for the test article.

⟨1191⟩ STABILITY CONSIDERATIONS IN DISPENSING PRACTICE

NOTE—Inasmuch as this chapter is for purposes of general information only, no statement in the chapter is intended to modify or supplant any of the specific requirements pertinent to Pharmacopeial articles, which are given elsewhere in this Pharmacopeia.

Aspects of drug product stability that are of primary concern to the pharmacist in the dispensing of medications are discussed herein.

Pharmacists should avoid ingredients and conditions that could result in excessive physical deterioration or chemical decomposition of drug preparations, especially when compounding (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩). The stability and clinical effect of manufactured dosage forms can be greatly compromised by seemingly negligible alterations or inappropriate prescription compounding. Pharmacists should establish and maintain compounding conditions that include the ensuring of drug stability to help prevent therapeutic failure and adverse responses.

Stability—Stability is defined as the extent to which a product retains, within specified limits, and throughout its period of storage and use (i.e., its shelf-life), the same properties and characteristics that it possessed at the time of its manufacture. Five types of stability generally recognized are shown in the accompanying table.

Criteria for Acceptable Levels of Stability

Type of Stability	Conditions Maintained Throughout the Shelf Life of the Drug Product
Chemical	Each active ingredient retains its chemical integrity and labeled potency, within the specified limits.
Physical	The original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability, are retained.
Microbiological	Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits.
Therapeutic	The therapeutic effect remains unchanged.
Toxicological	No significant increase in toxicity occurs.

FACTORS AFFECTING PRODUCT STABILITY

Each ingredient, whether therapeutically active or pharmaceutically necessary, can affect the stability of drug substances and dosage forms. The primary environmental factors that can reduce stability include exposure to adverse temperatures, light, humidity, oxygen, and carbon dioxide. The major dosage form factors that influence drug stability include particle size (especially in emulsions and suspensions), pH, solvent system composition (i.e., percentage of "free" water and overall polarity), compatibility of anions and cations, solution ionic strength, primary container, specific chemical additives, and molecular binding and diffusion of drugs and excipients. In dosage forms, the following reactions usually cause loss of active drug content, and they usually do not provide obvious visual or olfactory evidence of their occurrence.

Hydrolysis

Esters and β -lactams are the chemical bonds that are most likely to hydrolyze in the presence of water. For example, the acetyl ester in aspirin is hydrolyzed to acetic acid and salicylic acid in the presence of moisture, but in a dry environment the hydrolysis of aspirin is negligible. The aspirin hydrolysis rate increases in direct proportion to the water vapor pressure in an environment.

The amide bond also hydrolyzes, though generally at a slower rate than comparable esters. For example, procaine (an ester) will hydrolyze upon autoclaving, but procainamide will not. The amide or peptide bond in peptides and proteins varies in the lability to hydrolysis.

The lactam and azomethine (or imine) bonds in benzodiazepines are also labile to hydrolysis. The major chemical accelerators or catalysts of hydrolysis are adverse pH and specific chemicals (e.g., dextrose and copper in the case of ampicillin hydrolysis).

Epimerization

Members of the tetracycline family are most likely to incur epimerization. This reaction occurs rapidly when the dissolved drug is exposed to a pH of an intermediate range (higher than 3), and it results in the steric rearrangement of the dimethylamino group. The epimer of tetracycline, epitetracycline, has little or no antibacterial activity.

Decarboxylation

Some dissolved carboxylic acids, such as *p*-aminosalicylic acid, lose carbon dioxide from the carboxyl group when heated. The resulting product has reduced pharmacological potency.

β-Keto decarboxylation can occur in some solid antibiotics that have a carbonyl group on the β-carbon of a carboxylic acid or a carboxylate anion. Such decarboxylations will occur in the following antibiotics: carbenicillin sodium, carbenicillin free acid, ticarcillin sodium, and ticarcillin free acid.

Dehydration

Acid-catalyzed dehydration of tetracycline forms epianhydrotetracycline, a product that both lacks antibacterial activity and causes toxicity.

Oxidation

The molecular structures most likely to oxidize are those with a hydroxyl group directly bonded to an aromatic ring (e.g., phenol derivatives such as catecholamines and morphine), conjugated dienes (e.g., vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (e.g., flavorings). Products of oxidation usually lack therapeutic activity. Visual identification of oxidation, for example, the change from colorless epinephrine to its amber colored products, may not be visible in some dilutions or to some eyes.

Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metal ions (e.g., copper and iron), and exposure to oxygen and UV illumination. The latter two causes of oxidation justify the use of antioxidant chemicals, nitrogen atmospheres during ampul and vial filling, opaque external packaging, and transparent amber glass or plastic containers.

Photochemical Decomposition

Exposure to, primarily, UV illumination may cause oxidation (photo-oxidation) and scission (photolysis) of covalent bonds. Nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions.

Ionic Strength

The effect of the total concentration of dissolved electrolytes on the rate of hydrolysis reactions results from the influence of ionic strength on interionic attraction. In general, the hydrolysis rate constant is inversely proportional to the ionic strength with oppositely charged ions (e.g., drug cation and excipient anions) and directly proportional to the ionic strength with ions of like charge. A reaction that produces an ion of opposite charge to the original drug ion because of the increasing ionic strength, can increase the drug hydrolysis rate as the reaction proceeds. High ionic strength of inorganic salts can also reduce the solubility of some other drugs.

pH Effect

The degradation of many drugs in solution accelerates or decelerates exponentially as the pH is decreased or increased over a specific range of pH values. Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions. A drug solution or suspension, for example, may be stable for days, weeks, or even years in its original formulation, but when mixed with another liquid that changes the pH, it degrades in minutes or days. It is possible that a pH change of only 1 unit (e.g., from 4 to 3 or 8 to 9) could decrease drug stability by a factor of 10 or greater.

A pH buffer system, which is usually a weak acid or base and its salt, is a common excipient used in liquid preparations to maintain the pH in a range that minimizes the drug degradation rate. The pH of drug solutions may also be either buffered or adjusted to achieve drug solubility. For example, pH in relation to pKa controls the fractions of the usually more soluble ionized and less soluble nonionized species of weak organic electrolytes.

The influence of pH on the physical stability of two phase systems, especially emulsions, is also important. For example, intravenous fat emulsion is destabilized by acidic pH.

Interionic (Ion^{N+}–Ion^{N-}) Compatibility

The compatibility or solubility of oppositely charged ions depends mainly on the number of charges per ion and the molecular size of the ions. In general, polyvalent ions of opposite charge are more likely to be incompatible. Thus, an incompatibility is likely to occur upon the addition of a large ion with a charge opposite to that of the drug.

Solid State Stability

Solid state reactions are relatively slow; thus, stability of drugs in the solid state is rarely a dispensing concern. The degradation rate of dry solids is usually characterized by first-order kinetics or a sigmoid curve. Therefore, solid drugs with lower melting point temperatures should not be combined with other chemicals that would form a eutectic mixture.

When moisture is present, the solid drug decomposition may change to zero-order chemical kinetics because the rate is controlled by the relatively small fraction of the drug that exists in a saturated solution, which is located (usually imperceptibly) at the surface or in the bulk of the solid drug product.

Temperature

In general, the rate of a chemical reaction increases exponentially for each 10° increase in temperature. This relationship has been observed for nearly all drug hydrolysis and some drug oxidation reactions. The actual factor of rate increase depends on the activation energy of the particular reaction. The activation energy is a function of the specific reactive bond and the drug formulation (e.g., solvent, pH, additives). As an example, consider a hydrolyzable drug that is exposed to a 20° increase in temperature, such as that from cold to controlled room temperature (see *General Notices and Requirements*). The shelf life of the drug at controlled room temperature should be expected to decrease to one-fourth to one-twenty-fifth of its shelf life under refrigeration.

The pharmacist should also be aware that inappropriately cold temperatures may cause harm. For example, refrigeration may cause extreme viscosity in some liquid drugs and cause supersaturation in others. Freezing may either break or cause a large increase in the droplet size of emulsions; it can denature proteins; and in rare cases, it can cause less soluble polymorphic states of some drugs to form.

STABILITY STUDIES IN MANUFACTURING

The scope and design of a stability study vary according to the product and the manufacturer concerned. Ordinarily the formulator of a product first determines the effects of temperature, light, air, pH, moisture, trace metals, and commonly used excipients or solvents on the active ingredient(s). From this information, one or more formulations of each dosage form are prepared, packaged in suitable containers, and stored under a variety of environmental conditions, both exaggerated and normal. At appropriate time intervals, samples of the product are assayed for potency by use of a stability-indicating method, observed for physical changes, and, where applicable, tested for sterility and or for resistance to microbial growth and for toxicity and bioavailability. Such a study, in combination with clinical and toxicological results, enables the manufacturer to select the optimum formulation and container and to assign recommended storage conditions and an expiration date for each dosage form in its package.

Responsibility of Pharmacists

Pharmacists help to ensure that the products under their supervision meet acceptable criteria of stability by (1) dispensing oldest stock first and observing expiration dates, (2) storing products under the environmental conditions stated in the individual monographs, labeling, or both, (3) observing products for evidence of instability, (4) properly treating and labeling products that are repackaged, diluted, or mixed with other products, (5) dispensing in the proper container with the proper closure, and (6) informing and educating patients concerning the proper storage and use of the products, including the disposition of outdated or excessively aged prescriptions.

ROTATION OF STOCK AND OBSERVANCE OF EXPIRATION DATES

Proper rotation of stock is necessary to ensure the dispensing of suitable products. A product that is dispensed infrequently should be closely monitored so that old stocks are given special attention, particularly with regard to expiration dates. The manufacturer can guarantee the quality of a product up to the time designated as its expiration date only if the product has been stored in the original container under recommended storage conditions.

STORAGE UNDER RECOMMENDED ENVIRONMENTAL CONDITIONS

In most instances, the recommended storage conditions are stated on the label, in which case it is imperative to adhere to those conditions. They may include a specified temperature range or a designated storage place or condition (e.g., "refrigerator," or "controlled room temperature") as defined in the *General Notices*. Supplemental instructions, such as a direction to protect the product from light, also should be followed carefully. Where a product is required to be protected from light and is in a clear or translucent container enclosed in an opaque outer covering, such outer covering is not to be removed and discarded until the contents have been used. In the absence of specific instructions, the product should be stored at controlled room

temperature (see *Storage Temperature* in the *General Notices*). The product should be stored away from locations where excessive or variable heat, cold, or light prevails, such as those near heating pipes or fluorescent lighting.

OBSERVING PRODUCTS FOR EVIDENCE OF INSTABILITY

Loss of potency usually results from a chemical change, the most common reactions being hydrolysis, oxidation-reduction, and photolysis. Chemical changes may also occur through interaction between ingredients within a product, or rarely between product and container. An apparent loss of potency in the active ingredient(s) may result from diffusion of the drug into, or its combination with, the surface of the container-closure system. An apparent gain in potency usually is caused by solvent evaporation or by leaching of materials from the container–closure system.

The chemical potency of the active ingredient(s) is required to remain within the limits specified in the monograph definition. Potency is determined by means of an assay procedure that differentiates between the intact molecule and its degradation products. Chemical stability data should be available from the manufacturer. Although chemical degradation ordinarily cannot be detected by the pharmacist, excessive chemical degradation sometimes is accompanied by observable physical changes. In addition, some physical changes not necessarily related to chemical potency, such as change in color and odor, formation of a precipitate, or clouding of solution, may serve to alert the pharmacist to the possibility of a stability problem. It should be assumed that a product that has undergone a physical change not explained in the labeling may also have undergone a chemical change, and such a product is never to be dispensed. Excessive microbial growth, contamination, or both, may also appear as a physical change. A gross change in a physical characteristic such as color or odor is a sign of instability in any product. Other common physical signs of deterioration of dosage forms include the following.

Solid dosage forms: Many solid dosage forms are designed for storage under low-moisture conditions. They require protection from environmental water and therefore should be stored in tight containers (see *Containers* in the *General Notices*) or in the container supplied by the manufacturer. The appearance of fog or liquid droplets, or clumping of the product, inside the container signifies improper conditions. The presence of a desiccant inside the manufacturer's container indicates that special care should be taken in dispensing. Some degradation products, for example, salicylic acid from aspirin, may sublime and be deposited as crystals on the outside of the dosage form or on the walls of the container.

Hard and soft gelatin capsules: Since the capsule formulation is encased in a gelatin shell, a change in gross physical appearance or consistency, including hardening or softening of the shell, is the primary evidence of instability. Evidence of release of gas, such as a distended paper seal, is another sign of instability.

Uncoated tablets: Evidence of physical instability in uncoated tablets may be shown by excessive powder and/or pieces (i.e., crumbling as distinct from breakage) of tablet at the bottom of the container (from abraded, crushed, or broken tablets); cracks or chips in tablet surfaces; swelling; mottling; discoloration; fusion between tablets; or the appearance of crystals that obviously are not part of the tablet itself on the container walls or on the tablets.

Coated tablets: Evidence of physical instability in coated tablets is shown by cracks, mottling, or tackiness in the coating and the clumping of tablets.

Dry powders and granules: Dry powders and granules that are not intended for constitution into a liquid form in the original container may cake into hard masses or change color, which may render them unacceptable.

Powders and granules intended for constitution as suspensions: Dry powders and granules intended for constitution into solutions or suspensions require special attention. Usually such forms are antibiotics or vitamins that are particularly sensitive to moisture. Since they are always dispensed in the original container, they generally are not subject to contamination by moisture. However, an unusual caked appearance necessitates careful evaluation, and the presence of a fog or liquid droplets inside the container generally renders the preparation unfit for use. Presence of an objectionable odor also may be evidence of instability.

Effervescent tablets, granules, and powders: Effervescent products are particularly sensitive to moisture. Swelling of the mass or development of gas pressure is a specific sign of instability, indicating that some of the effervescent action has occurred prematurely.

Liquid dosage forms: Of primary concern with respect to liquid dosage forms are homogeneity and freedom from excessive microbial contamination and growth. Instability may be indicated by cloudiness or precipitation in a solution, breaking of an emulsion, nonresuspendable caking of a suspension, or organoleptic changes. Microbial growth may be accompanied by discoloration, turbidity, or gas formation.

Solutions, elixirs, and syrups: Precipitation and evidence of microbial or chemical gas formation are the two major signs of instability.

Emulsions: The breaking of an emulsion (i.e., separation of an oil phase that is not easily dispersed) is a characteristic sign of instability; this is not to be confused with creaming, an easily redispersible separation of the oil phase that is a common occurrence with stable emulsions.

Suspensions: A caked solid phase that cannot be resuspended by a reasonable amount of shaking is a primary indication of instability in a suspension. The presence of relatively large particles may mean that excessive crystal growth has occurred.

Tinctures and fluidextracts: Tinctures, fluidextracts, and similar preparations usually are dark because they are concentrated, and thus they should be scrutinized carefully for evidence of precipitation.

Sterile liquids: Maintenance of sterility is of course critical for sterile liquids. The presence of microbial contamination in sterile liquids usually cannot be detected visually, but any haze, color change, cloudiness, surface film, particulate or flocculent

matter, or gas formation is sufficient reason to suspect possible contamination. Clarity of sterile solutions intended for ophthalmic or parenteral use is of utmost importance. Evidence that the integrity of the seal has been violated on such products should make them suspect.

Semisolids (creams, ointments, and suppositories): For creams, ointments, and suppositories, the primary indication of instability is often either discoloration or a noticeable change in consistency or odor.

Creams: Unlike ointments, creams usually are emulsions containing water and oil. Indications of instability in creams are emulsion breakage, crystal growth, shrinking due to evaporation of water, and gross microbial contamination.

Ointments: Common signs of instability in ointments are a change in consistency and excessive "bleeding" (i.e., separation of excessive amounts of liquid) and formation of granules or grittiness.

Suppositories: Excessive softening is the major indication of instability in suppositories, although some suppositories may dry out and harden or shrivel. Evidence of oil stains on packaging material should warn the pharmacist to examine individual suppositories more closely by removing any foil covering. As a general rule (although there are exceptions), suppositories should be stored in a refrigerator (see *Storage Temperature* in the *General Notices*).

PROPER TREATMENT OF PRODUCTS SUBJECTED TO ADDITIONAL MANIPULATIONS

In repackaging, diluting a product or mixing it with another product, the pharmacist may become responsible for its stability.

Repackaging: In general, repackaging is inadvisable. However, if repackaging is necessary, the manufacturer should be consulted concerning potential problems. In the filling of prescriptions, it is essential that suitable containers be used. Appropriate storage conditions and, when appropriate, an expiration date and beyond use date should be indicated on the label of the prescription container. Single-unit packaging calls for care and judgment and for strict observance of the following guidelines: (1) use appropriate packaging materials, (2) if stability data on the new package are not available, repackage at any one time only sufficient stock for a limited time, (3) include on the unit-dose label a lot number and an appropriate beyond-use date, (4) if a sterile product is repackaged from a multiple-dose vial into unit-dose (disposable) syringes, discard the latter if not used within 24 hours, unless data are available to support longer storage, (5) if quantities are repackaged in advance of immediate need, maintain suitable repackaging records showing name of manufacturer, lot number, date, and designation of persons responsible for repackaging and for checking (see *General Notices*), (6) if safety closures are required, use container closure systems that ensure compliance with compendial and regulatory standards for storage.

Dilution or mixing: If a product is diluted, or if two products are mixed, the pharmacist should observe good professional and scientific procedures to guard against incompatibility and instability. For example, tinctures such as those of belladonna and digitalis contain high concentrations of alcohol to dissolve the active ingredient(s), and they may develop a precipitate if they are diluted or mixed with aqueous systems. Pertinent technical literature and labeling should be consulted routinely; it should be current literature, because at times formulas are changed by the manufacturer. If a particular combination is commonly used, consultation with the manufacturer(s) is advisable. Since the chemical stability of extemporaneously prepared mixtures is unknown, the use of such combinations should be discouraged; if such a mixture involves an incompatibility, the pharmacist might be responsible. Oral antibiotic preparations constituted from powder into liquid form should never be mixed with other products.

Combining parenteral products necessitates special care, particularly in the case of intravenous solutions, primarily because of the route of administration. This area of practice demands the utmost in care, aseptic technique, judgment, and diligence. Because of potential unobservable problems with respect to sterility and chemical stability, all extemporaneous parenteral preparations should be used within 24 hours unless data are available to support longer storage.

INFORMING AND EDUCATING THE PATIENT

As a final step in meeting responsibility for the stability of drugs dispensed, the pharmacist is obligated to inform the patient about the proper storage conditions (for example, in a cool, dry place—not in the bathroom) for both prescription and nonprescription products, and to suggest a reasonable estimate of the time after which the medication should be discarded. When beyond-use dates are applied, the pharmacist should emphasize to the patient that the dates are applicable only when proper storage conditions are observed. Patients should be encouraged to clean out their drug storage cabinets periodically.

⟨1195⟩ SIGNIFICANT CHANGE GUIDE FOR BULK PHARMACEUTICAL EXCIPIENTS

BACKGROUND

This general information chapter was derived from an international guidance on the evaluation of the significance of changes involving the manufacture of bulk pharmaceutical excipients. It is intended to assist excipient manufacturers in determining the need for informing the excipient user and regulatory authorities about the nature of the change.

The chapter provides minimum recommendations when considering the effect of a change in the manufacturing process on the excipient. When deciding how to use this chapter, each manufacturer must consider how it may apply to that manufacturer's product and processes. The diversity of excipients means that some principles of this chapter may not be applicable to certain products and processes.

This chapter is divided into several sections. The first section provides the general guidance necessary for evaluating a change and determining the necessity of informing the user and/or regulatory authorities. One section provides criteria for determining whether a change will involve a significant risk. Also included is a decision tree that is useful in considering the potential effect of a change on excipient performance.

INTRODUCTION

Purpose

This document is meant to establish uniform considerations for evaluating the significance of changes involving the manufacture of bulk pharmaceutical excipients (BPEs). The purpose of the evaluation is to determine the need for informing the excipient user and regulatory authorities about the nature of the change.

Scope

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients [referred to throughout this document as "excipient(s)"] intended for use in human drugs, veterinary drugs, and biologics. The principles set forth here must be applied once it has been determined that a chemical is intended for use as a component of a drug product. As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product should increase and should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩ should be applied and maintained. Judgment, based on risk analysis and a thorough knowledge of the process, is required to determine from which processing step the GMPs should be implemented.

Principles Adopted

This chapter should be of international application, bearing in mind that pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. It provides minimum recommendations when considering the impact of a change on the excipient. As an international guidance document, it cannot specify all national legal requirements nor cover in detail the particular characteristics of every excipient.

When considering how to use this chapter, each manufacturer should consider how it may apply to that manufacturer's product and processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes. The terminology "should" and "it is recommended" do not necessarily mean "must" as used in the application of this chapter.

Excipients may contain minor components that are known to be or might be necessary for the correct functioning of the excipient. The presence of these "essential concomitant components" in the excipient should not be construed as undesirable. These concomitant components are not considered part of the impurity profile, but should be considered separately. Water may be a concomitant component in some excipients, but may be included in the impurity profile for others. (See *Impurity Profile* in *Appendix 3* for more information.)

Layout

This chapter is divided into several sections. The first section provides a background discussion necessary for evaluating a change and determining the necessity of informing the user and/or regulatory authorities. A second section provides criteria for determining the risk that a change will be significant, including guidance on development of an impurity profile. Also included are the following: The *Glossary* contains terms and definitions used in all sections of this document. *Appendix 1* includes

some examples of changes that would be classified into each of the three risk levels. *Appendix 2* provides a decision tree useful in considering the potential impact of a change on excipient performance. *Appendix 3* delineates the development of an impurity profile.

GENERAL GUIDANCE

Differentiation of Excipient Manufacture

Evaluating the impact of a change in the manufacture of an excipient could be more difficult than that for an active pharmaceutical ingredient (API). Although the API is seldom used for more than a handful of therapeutic purposes, the BPE is often used with a broad range of active ingredients and in a diverse range of finished dosage forms. Whereas the API is typically of high purity and well characterized by the quality control and analytical laboratory, the BPE is often a natural substance, mixture, or polymer, the chemical and physical properties of which are more difficult to quantify. For a more thorough discussion of GMPs that apply to excipient manufacture, see the general information chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* 〈1078〉.

Definition of Significant Change

Any change by the manufacturer of an excipient that alters an excipient's physical or chemical property outside the established limits, or that is likely to alter the excipient performance in the dosage form, is considered significant. Such changes may necessitate notifying the local regulatory authority if required. Regardless of whether there is a regulatory requirement, the manufacturer has an obligation to notify its users of a significant change so that the user can evaluate the impact of the change on the user's products. It is suggested that unless there is clear indication from evaluation of the change that it is not significant as stipulated by this general chapter, the pharmaceutical user should be notified.

The types of change that are considered here are changes to the following: site, scale, equipment, process, packaging and labeling, and specification (including raw materials).

The requirement for evaluating the impact of change on the excipient begins, at a minimum, with the raw materials for the first processing step from where GMP compliance begins. GMP requirements increase as the manufacturing process progresses. Thus, at some logical processing step, usually well before the final finishing operation, appropriate GMPs should be imposed and maintained throughout the remainder of the process. Methods such as HACCP (Hazard Analysis and Critical Control Point), FEMA (Failure Effects Mode Analysis), or a detailed process flow diagram may be used to identify the unit operations, required equipment, stages at which various substances are added, key steps in the process, critical parameters (time, temperature, pressure, etc.), and necessary monitoring points. Judgment, based on risk analysis and a thorough knowledge of the process, is required to determine at which processing step the GMP should be implemented.[1]

It is important to give careful consideration to any processing changes that occur after the excipient has been synthesized or isolated but prior to packaging. However, it must be recognized that a change made earlier in the process can result in a change in the excipient functionality, and it is recommended that such changes also be considered.

SIGNIFICANT CHANGE

Evaluation Criteria

These criteria, in the form of questions, are presented for consideration when evaluating the impact of a change relating to excipient manufacture:

1. Has there been a change in the chemical properties or composition of the excipient as a result of the change?
2. Has there been a change in the physical properties of the excipient as a result of the change?
3. Has there been a change in the "essential concomitant components" profile for the excipient as a result of the change?
4. Has there been a change in the functionality of the excipient as a result of the change?
5. Where applicable, has the moisture level changed?
6. Where applicable, has the bioburden changed?
7. Has there been a change in the origin of any raw materials or contact packaging?

An affirmative answer to any of these questions indicates that the impact of the change on the excipient may lead to changes in its performance in the dosage form.

It is important to provide objective criteria for evaluating when a change has occurred in an excipient property or composition, in the essential concomitant components profile, in biological origin, or in its functionality. This enables the BPE manufacturer to evaluate the significance of the change on the excipient for the purpose of notifying the regulatory authorities and/or the user.

[1] See chapter 〈1078〉, *GMPs For Excipients.*

Determination of Significance

Criterion 1: Evaluation of the chemical properties or composition of an excipient should include, at a minimum, all monograph and manufacturer specification parameters. A comparison of these test results for the excipient before and after a change should be done to determine if there is a statistically significant difference.

Criterion 2: Physical properties should be considered based upon the physical form of the excipient and its functionality as known or as used by the end users. A physical property that is part of a mutually agreed-upon specification between the manufacturer and end user should also be evaluated. For example, a manufacturer of an excipient powder should consider measuring the impact of changes on such physical parameters as bulk density, surface area, particle shape, and particle size distribution. Liquid excipients might be evaluated for changes in their pH and viscosity. For all polymeric excipients, the effect of a change on a physical property, such as molecular weight distribution, should be considered.

Criterion 3: Objective criteria are also necessary when considering changes to the "essential concomitant components" profile for an excipient as a result of changes. The profile, as noted in *Appendix 3*, contains the following:

- identified organic impurities,
- unidentified organic impurities at or above 0.10%, whether specified or not,[2]
- residual solvents, and
- inorganic impurities

The feasibility of developing an impurity profile varies with the composition and origin of the excipient. It is important to note that identifying and quantifying impurities in some excipients are extremely difficult. Thus, an excipient manufacturer may not have developed an impurity profile. In that case, it is important for excipient manufacturers to either document their efforts to identify and quantify the impurities that may be present to justify their limited results or to justify other means by which changes may be evaluated.

The significance of the change can be determined by comparing the impurity profile of the pre-change material with that of the post-change product. Therefore, once the profile has been developed, it should be reassessed following changes to the process. An impurity should be monitored as part of the profile if it is present at or greater than 0.10%, if it has an established physiological effect, or if it is known to be unsafe at a lower level.

The content of the impurity profile varies with the nature of the excipient, the raw materials used in its manufacture, and its chemical composition. Where possible, changes are considered significant whenever a new impurity is introduced at the 0.10% concentration or higher, or when an impurity previously present at or greater than 0.10% disappears. Changes to the quantity of an existing impurity specified in a monograph and reported on the Certificate of Analysis (COA) should be treated as a chemical property for the purposes of this evaluation.

Changes in the levels of residual solvents should be considered when determining the significance of change. See *Residual Solvents* ⟨467⟩ for details.

Criterion 4: Objective criteria for evaluating changes to excipient functionality are desirable. However, the nature of this type of study can vary broadly based upon the excipient and its application in the dosage form. It must also be recognized that the excipient manufacturer does not always know each use of the excipient. Therefore this chapter cannot provide objective criteria for this study but stresses the importance of such a consideration by the manufacturer. If there is the potential that the functionality of the excipient may be affected by the change, users should be notified and material provided upon request so that they can determine the impact of the change in their finished pharmaceutical products.

Criterion 5: Often the excipient contains moisture, the presence of which can have an impact on excipient performance in the preparation of the pharmaceutical dosage form. Therefore a change in the moisture level beyond the range typical of production, even though within the compendial or specification limit, can affect its stability and/or end use.

Criterion 6: Change in the processing steps, raw materials, or equipment can adversely affect control of microorganisms in the excipient. Therefore the effect of the change on the bioburden should be evaluated, particularly for excipients susceptible to microbial growth.

Criterion 7: Change in the origin of a raw material or contact packaging can result in a change to the other six change criteria. Change in origin can involve the country of origin, geological origin, or species of origin for the raw material.

A change in the country of origin of a raw material or contact packaging material can affect the status of the excipient as it relates to the potential presence of bovine spongiform encephalopathies (BSEs), transmissible spongiform encephalopathy (TSE) material, or genetically modified organisms (GMOs). The country of origin of animal raw material, or of components used in the manufacture of the raw material, can result in noncompliance with relevant TSE regulations.[3,4] Current information on BSE and related diseases can be accessed on the United States Department of Agriculture (USDA) website (usda.gov).

A change in the geological origin of a mineral-based excipient can alter the composition of the excipient. Geological formations containing the same mineral can differ in their chemical composition, crystalline structure, density, etc. A change in geo-

[2] It is recognized that while desirable, it may not be possible to achieve this for all excipients, particularly those of a more complex chemical nature, e.g. natural polymers, for which there may be no adequate means of determining related substances. However, the impurity profile documentation should demonstrate why this was not achievable.

[3] *European Pharmacopoeia*, General Text 5.2.8, *Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Medicinal Products.*

[4] U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Federal Register: November 4, 2003, Volume 68, Number 213 (Proposed Rules), 9 CFR Parts 93, 94, and 95, *Bovine Spongiform Encephalopathy; Minimal Risk Regions and Importation of Commodities.*

logical origin of raw material can affect the excipient's chemical or physical properties, the impurity profile, or excipient functionality.

A change to the species of origin for raw materials of either animal or vegetable origin can raise concern. Switching from one animal species to another can affect the status of the excipient as it relates to the presence of BSE or TSE material in the excipient, as noted above. Switching from animal-derived to plant-derived raw materials, although eliminating the issue of BSE or TSE material, raises the potential for the presence of plant-based allergenic material in the excipient. Switching from one plant species to another also can result in the possible presence of an allergen in the excipient. In addition to this issue with allergens, use of plant-derived raw materials can affect pharmaceutical manufacturers who have a concern about the presence of GMOs in the excipient.

Risk Levels

In the evaluation of the effect of changes on the excipient, it is recognized that even with objective criteria, some judgment may be necessary. To facilitate the decision as to the significance of a change and the likely effect on the dosage form, the types of changes are classified using three levels:

- Level 1: Minor Change
- Level 2: Might Be Significant
- Level 3: Always Significant

LEVEL 1: MINOR CHANGE

These changes are considered unlikely to affect the excipient's chemical or physical properties, impurity profile, or functionality. Such changes should be documented, but notifications to the users and regulatory authorities are not necessary.

LEVEL 2: MIGHT BE SIGNIFICANT

The effect of the change should be evaluated against *Criterion 1, Criterion 2,* and *Criterion 3* (chemical and physical properties and impurity profile) to determine its potential effect on excipient functionality. A change in the biological origin of a raw material should be considered with regard to TSE or GMO regulations. A Level 2 change should always be communicated to the users and regulatory authorities.

LEVEL 3: ALWAYS SIGNIFICANT

This type of change should always be communicated to the users and regulatory authorities. Shipment of the changed excipient to the user should not occur without consent from the user's company.

Protocol Design

There should be a written protocol for the evaluation of a change to determine whether it is significant. The protocol should describe the nature of the change, the reason it may be significant, the testing to be performed to evaluate the change, and the criteria for determining the significance. If the change is attributable to a new biological source for raw materials used in the manufacture of the excipient, it is recommended that the regulatory status of the raw material (i.e., BSE/TSE, GMO agents) be evaluated first. Then, where possible, the results from the testing of a minimum of 10 pre- and 3 post-change batches of excipient should be compared (see *Supporting Data*, below). If significant changes are seen, then an assessment of the significance should be made.

The manufacturer should test the excipient made after the change for all specification properties and compare the results to the historical data. A standard statistical test, such as a *t*-test of the means, should be used to compare the new data with the historical data. If when using an appropriate statistical analysis there is sufficient evidence that the populations are different at the 95% confidence interval, the change should be considered significant. As an additional check on consistency, it is also recommended that the new batch specification properties be plotted on standard Statistical Quality Control (SQC) control charts, along with standard batch results.

Supporting Data

It is preferable to use data to measure the effect of a change on the excipient. The comparison should begin with chemical and physical properties, followed, where appropriate, by moisture, bioburden, impurity profile, and functionality. The manufacturer should use good judgment on sample comparisons for the other evaluations.

Chemical and physical properties lend themselves to quantitative measurement. Often these properties are part of the specification for the excipient. As such there should be a large body of test data to use for the properties affected for comparison to the corresponding data of the excipient made after the change.

Equivalence of impurity profiles is shown by comparing the data for the pre-change and post-change batches. If the following conditions are met, there has been no significant change in the impurity profile. [NOTE—*Residual Solvents* ⟨467⟩ notes that under certain circumstances an impurity concentration below 0.10% may be of concern and the excipient manufacturer should take this under consideration.]

1. No new impurity is present at or above 0.10%, nor has an impurity at this level disappeared that was previously in the impurity profile.
2. Residual solvent and impurities remain within the 95% confidence interval of the mean of the batches produced before the change.

TYPES OF CHANGES

Site Change

A change in site can involve either the production or packaging of the excipient or its quality control testing. If the proposed manufacturing site was never used to produce the excipient, then the change poses a greater risk of altering the excipient's performance and is considered a *Level 3* change. If the proposed site was used for this purpose within the past year and the process, equipment, utilities, and raw materials are all unchanged, the risk is considered minor and thus a *Level 1* change. However, if the excipient was produced before at the proposed site with the same process, equipment, utilities, and raw materials more than 1 year ago, the risk is moderate and thus *Level 2*.

If the change involves the quality control laboratory, then the impact hinges on the test method. If the method remains the same, the change is a *Level 1*, provided a formal method transfer or validation is conducted. If the new lab uses a different analytical technique or analytical equipment, then the change should be evaluated more carefully, as required by a *Level 2* change.

Scale

Manufacturers often find ways to increase the scale of production. If the excipient is being scaled up from pilot to production, the change is likely to be significant and thus a *Level 3*. When the change in scale results from the use of new and larger, or smaller, production equipment using the same operating principle, which is often the case in batch processing, the change is a *Level 2*. If the existing equipment is optimized to increase capacity without altering the process, often found in continuous processing, the change is considered minor and treated as *Level 1* provided that a comparison of pre- and post-change data shows no statistically significant difference. However, careful consideration should be given to changes that are made that can clearly affect the properties of the excipient.

Equipment

The evaluation of equipment change concerns the issue of whether it is equivalent to the equipment it replaces. Generally, equipment that is a replacement in kind is considered a minor *Level 1* change. If the new equipment is not a replacement in kind but is included in the process validation, then the change is still a *Level 1*. Otherwise the change is considered *Level 3*.

Manufacturing Process

A change in process often involves changes to the processing instructions, such as target levels for such parameters as temperature, pressure, and flow rate; the raw materials to be used; the sequence of operating steps; and the operation to be performed, including reprocessing. As illustrated in the decision tree in *Appendix 2*, each type of process change can be further detailed.

If there is a change in a process parameter that is within the process validation, such as operating at a new target within the qualified range, then the change is a *Level 1*. However, if the process parameter is outside of the validation, then the change should be evaluated as a *Level 2*.

If minor changes are made to the processing steps, such as a small change outside of the validated range in the rate of addition of an ingredient, then the change is a *Level 2*. A major change, such as changing the point at which an ingredient is added to earlier or later in the process, is potentially significant and thus a *Level 3*.

Reprocessing of an excipient followed by a purification step, when not typical of the process, should be evaluated as a *Level 2* change. However, if no further purification of the bulk excipient occurs, this type of change is considered a *Level 3*.

Packaging and Labeling

These changes involve the package components meant for protection and distribution of the excipient. Any change in the package or packaging components such as the drum, box, liner, or tamper-evident seal that is a replacement in kind is a minor change *(Level 1)*. Replacement in kind applies to containers constructed of the same materials and sealed in a similar manner,

and liners made of the same components. Any change that is not a replacement in kind should be evaluated as a *Level 3*. Any change to labeling pertaining to the site of manufacture or testing, the biological origin, additives, or storage and handling conditions should be evaluated as a *Level 3*.

Specifications

Differences in raw materials can be further defined by the supplier used, their specifications, biological origin, country of origin for those derived from animals, or the addition to or removal of the raw material from the BPE process. If the new supplier provides its raw material against a specification essentially the same as that of the former supplier and the raw material method of manufacture is similar, the change is minor and treated as *Level 1*. However, if the specifications, biological origin, or country of origin changes, or the manufacturing process is different, then the change should be evaluated as potentially significant *(Level 2)*. Also, any change of source for an animal-origin material should be treated as a *Level 2* change, if the source is determined to be not from a risk country as codified in 9 CFR 94.18. Finally, if the change in raw material involves the addition or removal of an ingredient from the process to produce or preserve the BPE or is otherwise used to produce the bulk excipient, the change is likely to be significant *(Level 3)*. Similar consideration should be given for any change in origin of raw materials that results in a potential that the raw material might contain risk materials (i.e., BSE, TSE, allergens, or GMOs).

Changes are sometimes made to the excipient specification or the quality control test method. When changes are not the result of a monograph change, their significance should be evaluated. Such test or specification changes may be made to the excipient product or to the intermediate component. [NOTE—In some circumstances, relaxing the specification may lower the quality if the specification is for a significant property; therefore any change needs to be evaluated and its significance needs to be documented.]

Changes to an excipient specification or test method are *Level 3* changes. For example, adding a new specification parameter for the purpose of improving the quality of the excipient through lot selection is potentially a very significant *Level 3* change. If the specification change relaxes a specification parameter, the effect on excipient quality should be evaluated as a *Level 2* change. An example of a minor change is the additional testing of the excipient initiated with the sole purpose of further characterizing the material without altering its quality, and is a *Level 1* risk; however, notification is supported.

If a specification for a raw material from the same supplier(s) is made more stringent, then the change is unlikely to be significant *(Level 1)*, whereas if the specification becomes less stringent, then the change should be evaluated carefully *(Level 2)*.

When a change is made that either increases or maintains the level of process control in the manufacturing process, it should be treated as a *Level 1*. If the change in process control relaxes the control, then the effect should be carefully evaluated as *Level 2*. An illustrative example is pH control. If a new pH meter allows for more precise measurement, the process control is improved and the change falls under *Level 1*. However, if the pH control is relaxed by using a less precise measuring device, the change is treated as *Level 2*.

Multiple Changes

Multiple changes involve more than one change occurring simultaneously. The risk level for consideration of the impact of the changes should be the highest level for any single change. However, the effect of the totality of changes should also be assessed, because this may suggest that the overall risk is higher.

REPORTING REQUIREMENTS

Documentation

It is recommended that the evaluation of changes to the excipient be documented, regardless of the level of change. The report should indicate the basis for evaluating the effect of the change on the excipient, the significance of the data used in reaching the conclusion, and the actions taken.

Where appropriate, the process validation should be updated to reflect the changed process. This is clearly indicated where the evaluation has led to the conclusion that the change should be considered significant.

Notification

The user should be given as much advance notification of impending change as possible. For *Level 3* changes in particular, the user may require time to complete the evaluation of the impact of the change on their formulations. During this period the user may request inventory of the excipient produced before the change was made. The manufacturer should plan for the change with this eventuality in mind.

Regardless of the apparent level of the change, changes that are found to meet the definition of significant change resulting from the evaluation require user notification.

Regulatory authorities often require notification of significant changes involving the manufacture of excipients. Such notification should be done as required by the applicable authority.

GLOSSARY

Active Pharmaceutical Ingredient (API): Any substance or mixture of substances that is intended to be used in the manufacture of a drug (medicinal) product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.
Batch Process: A manufacturing process that produces the excipient from a discrete supply of the raw materials that are present before the completion of the reaction.
Bioburden: The nature and quantity of microorganisms present in the excipient.
Biological Origin: Defined as either animal origin or nonanimal origin, based on the source of the raw material used in the manufacture of the excipient, and also includes materials that potentially come into contact with equipment used in the manufacture of other materials with animal-derived or GMO-derived components.
Bovine Spongiform Encephalopathy (BSE): A pathological brain deterioration condition of cattle believed to be caused by a prion that can be transmitted to cause variant Creutzfeldt-Jakob disease (vCJD) in humans.
Bulk Pharmaceutical Excipient (BPE): See *Excipient*.
Chemical Property: A quality parameter that is measured by chemical or physicochemical test methods.
Concomitant Component: A substance found in an excipient that is not the intended chemical entity, but may be necessary for ensuring the proper performance of the excipient in its intended use, and is not an impurity or a foreign substance (formerly referred to as a minor component).
Confidence Interval: A range, calculated from sample data, within which a population parameter, such as the population mean, is expected to lie, with a given level of confidence.
Continuous Process: A manufacturing process that continually produces the excipient from a continuous supply of raw material.
Decision Tree: A visual presentation of the sequence of events that can occur, including decision points.
Drug Substance: See *Active Pharmaceutical Ingredient*.
Equipment: The implements used in the manufacture of an excipient.
Excipient: Any substance, other than the drug substance, in a drug product that has been appropriately evaluated for safety and is included in a drug delivery system to either aid the processing of the drug product during its manufacture; protect, support or enhance stability, bioavailability, or patient acceptability; assist in product identification; or enhance any other attribute of the overall safety and effectiveness of the drug product during storage or use.
Foreign Substance: A component that is present in the BPE but that is *not* introduced into the excipient as a consequence of its synthesis or purification and is not necessary to achieve the required functionality (formerly referred to as a contaminant).
Functionality: The set of performance criteria that the excipient is intended to meet when used in a formulation.
Genetically Modified Organism (GMO): Living organisms such as animals, plants, or microbes with an altered genetic makeup produced using a special set of technologies.
Impurity: A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.
Impurity Profile: A description of the impurities present in the excipient.
Mass Balance: The sum of the quantifiable material present in the excipient.
Packaging: The container and its components that hold the excipient for transport to the user.
Physical Property: A quality parameter that can be measured solely by physical means.
Physiological Effect: Any effect on the normal health of the human body.
Process: The set of operating instructions describing how the excipient is to be synthesized, isolated, purified, packaged, etc.
Process Parameter: A measurable operating condition.
Process Step: An instruction to the BPE manufacturing personnel directing that an operation be done.
Process Validation: A documented program that provides a high degree of assurance that a specific process will consistently produce a result that will meet predetermined acceptance criteria.
Raw Material: Any substance used in the production of an excipient, excluding packaging materials.
Replacement In Kind: Manufacturing equipment that uses the same operating principles and is of similar construction or packaging components made with the same materials of construction and sealed in a similar manner.
Reprocessing: Introduction of previously processed material that did not conform to standards or specifications back into the process and with repetition of one or more necessary steps that are part of the normal manufacturing process.
Residual Solvent: An organic volatile chemical that is used or produced in the manufacture of excipients. The residual solvent is not completely removed by practical manufacturing techniques.
Scale: An increase or decrease in the batch size in batch processing or the throughput capability for continuous processing, whether or not different equipment is used.
Significant Change: A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.
Site: A defined location of the equipment in which the excipient is manufactured. It may be within a larger facility. A change in site may be to a different part of the existing facility but in a different operational area or may be to a remote facility, including a contract manufacturer.

Solvent: A vehicle, other than water, used in the synthesis of the product that remains chemically unchanged.
Specification: The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.
Statistical Quality Control (SQC): The plotting of sequential test results to show their variation relative to the specification range and their normal variation.
Transmissible Spongiform Encephalopathy (TSE): Any agent that causes a symptomatic illness in animals or humans akin to BSE and variant Creutzfeldt-Jakob disease (vCJD), e.g., scrapie in sheep.

APPENDICES

Appendix 1: Change Levels

For guidance, examples of changes that typically would be classified into the three change levels are provided.

LEVEL 1

1. A processing parameter changed to a new set point that is within the process validation.
2. Use of alternate equipment that is listed as an alternate in a regulatory document (i.e., Drug Master File).
3. Use of equipment that is a replacement in kind. This is typically new equipment that uses the same operating principles as the equipment replaced.
4. Revision to a specification for one of the excipient's raw materials that involves more stringent quality or conformance to additional pharmacopeias.
5. Addition of a test parameter or tightening of an existing parameter to an excipient specification that is used for informational purposes only. This is not used for quality improvement or control purposes.
6. Improved environmental control to prevent cross-contamination of the excipient. An example of this is an improved packaging room or additional segregation of manufacturing equipment.

LEVEL 2

1. A processing parameter changed to a new set point that is outside of the process validation.
2. A site change returning the manufacture of an excipient to a site previously used for this purpose more than 1 year ago.
3. Process control that is outside the normal limits of variability. An example of this is new process control equipment for control of excipient properties not previously controlled that create process adjustments.
4. A change in the handling, storage, or delivery of the excipient. An example of a handling change is the movement of a powder with new powder-conveying equipment. The storage of the excipient in bulk versus the shipping container is illustrative of a change in storage. The delivery of the excipient in temperature-controlled trucks versus uncontrolled trucks exemplifies a change in delivery but not vice versa.
5. A change in container size or shape.

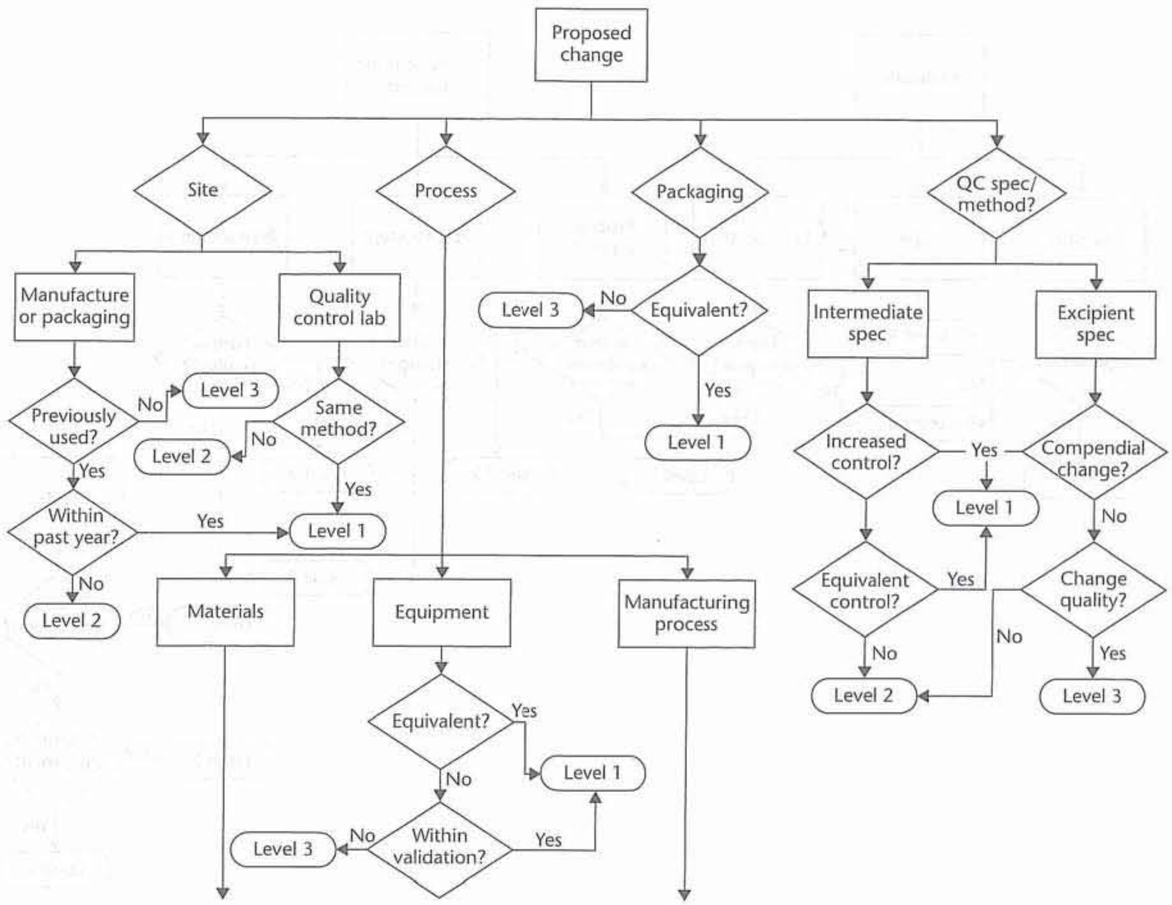
LEVEL 3

1. Addition or removal of a chemical entity from the manufacturing process. An example would be the addition or removal of a preservative agent, buffering agent, stabilizer, or catalyst.
2. Manufacture at a new site never used for this purpose.
3. Revision to a sales specification made for the purpose of improving the quality of the excipient either through improved process control or lot selection.
4. Use of a new package, such as a drum of a different construction (i.e., plastic versus steel).
5. Revision of the product label.
6. Revision of the tamper-evident seal.
7. A change to the stated shelf life or retest interval.

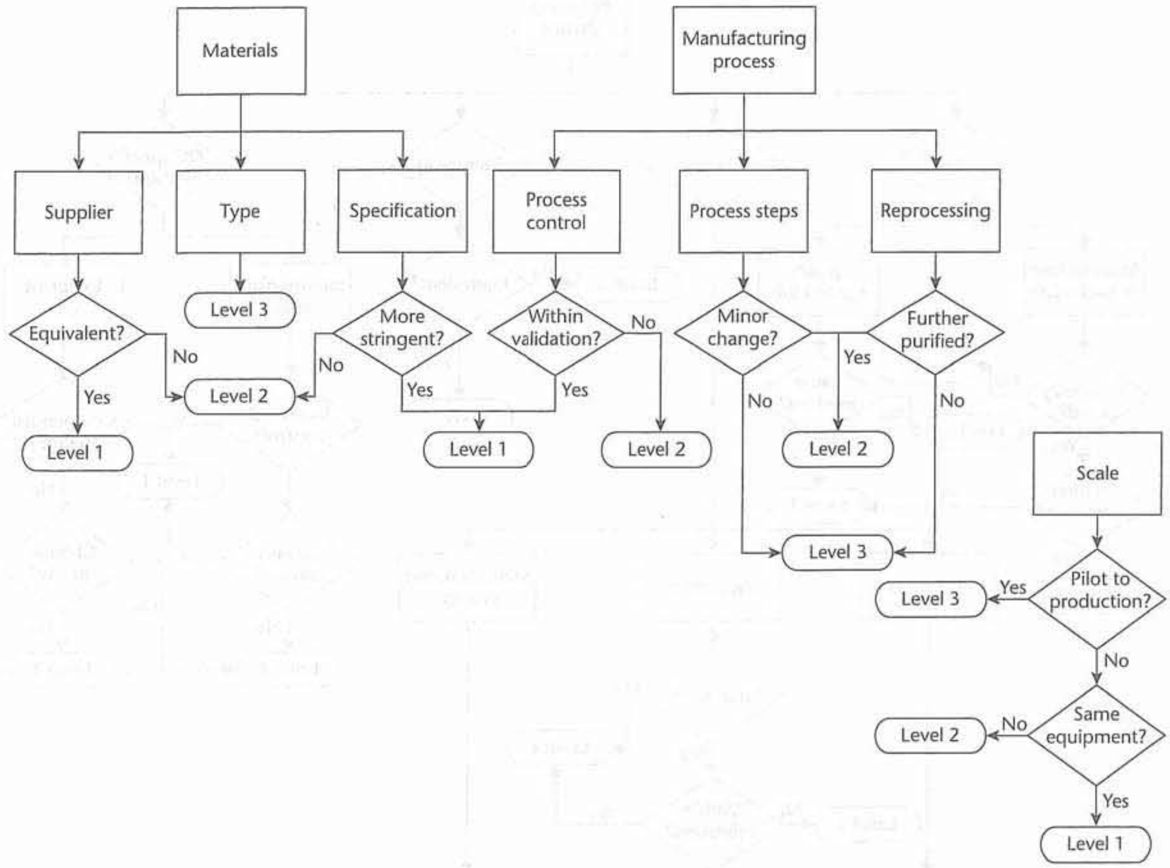
Appendix 2: Decision Tree

A decision tree has been developed to aid in classifying the change into levels. The diagram begins with the proposed change and guides the BPE maker to an indication of the likelihood that the change will affect the excipient user. The decision tree classifies the types of change that occur in excipient manufacture as involving the site of manufacture, the processing steps, packaging, and testing and quality control.

Appendix 2: Decision tree



Appendix 2 : Decision tree (continued)



Appendix 3: Impurity Profile

DEFINITION OF IMPURITY PROFILE

The impurity profile of an excipient may be defined as a description of the impurities present in a typical lot of excipient produced by a given manufacturing process. The impurity profile includes the identity of each major impurity or an appropriate qualitative description, such as peak retention time (if unidentified), the quantity of impurity observed expressed as a range, and the classification, as discussed below, of each identified impurity. Excipients frequently function because they are not "pure". That is to say that often there are concomitant components that are necessary for the correct functioning of the excipient. These essential concomitant components should not be considered as part of the impurity profile but should be evaluated separately, if possible.

The composition of the impurity profile is dependent upon such variables as the raw materials, solvents, reagents, catalysts, and manufacturing process used in the excipient's manufacture. Foreign substances, such as manufacturing aids that can be present in the excipient, should be controlled to a level that is unobjectionable.[5]

It is recognized that the presence of essential concomitant components is important to the performance of the excipient in the drug product. Therefore, the presence of these essential concomitant components in the excipient should neither be construed as being undesirable nor be confused with the presence of foreign substances or impurities.

It should be noted that in some excipients, water may be an essential concomitant component, necessary to achieve the desired functionality. For other excipients, water may be included in the impurity profile, if appropriate, and should be classified as an inorganic impurity in such circumstances.

USE OF THE IMPURITY PROFILE

The impurity profile, as used in this chapter, is meant to help determine the significance of a change. Impurities should be profiled by the excipient manufacturer if possible. This may be accomplished through knowledge of the starting materials and

[5] Current USP General Notices.

manufacturing process and subsequent application of validated analytical testing to provide a qualitative and/or quantitative result of the impurity profile.

PROCEDURE FOR DEVELOPMENT OF AN IMPURITY PROFILE

Because of the diverse nature of substances that may be incorporated as pharmaceutical excipients, including highly complex mixtures from animal, botanical, mineral, and/or synthetic sources, differing approaches to characterizing their properties may be required. It is recognized that the development of an impurity profile may not be technically feasible for certain excipients. In such cases, the manufacturer should document what method is being used to monitor the excipient for the effect of changes as noted in this chapter in *Evaluation Criteria* and *Determination of Significance* in *Significant Change*.

CLASSIFICATION OF IMPURITIES

Excipient impurities are classified as follows.

Organic Impurities: Any organic material that arises during the manufacturing process that is not listed as the intended excipient in the monograph or specification and is not a concomitant component or foreign substance. This may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts.

Inorganic Impurities: Any inorganic material that arises during the manufacturing process that is not listed as the intended excipient in the monograph or specification and is not a concomitant component or foreign substance. This may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts.

Residual Solvents: Solvents resulting from the incomplete removal of organic or inorganic liquids, regardless of the source. See *Residual Solvents* ⟨467⟩ for details. Note that the limits specified apply to the drug product as considered in *Option 2* and to the excipient as in *Option 1*. It should be noted that a residual solvent can also be classified as a concomitant component but still must be considered.

IMPURITY PROFILE

The characterization of the impurity profile of an excipient should be attempted by the manufacturer, where possible, by taking into account the manufacturing process and potential impurities anticipated as a consequence. A sensible approach includes control of all impurities that have known toxicological characteristics. The limits of these impurities may be based upon the usage of the drug product when so informed by the user and should comply with the requirements of ICH Q3B(R) *Impurities in Drug Products* and of *Residual Solvents* ⟨467⟩.

For the purpose of developing an impurity profile, excipients may be classified as those where purity can be directly measured and those where purity cannot be directly measured. Examples of the former are excipients whose monograph or specification includes a requirement for purity. Polymers or derivatives of naturally occurring products are often examples of excipients where purity cannot be directly measured.

The material to be used for the development of the impurity profile should be sampled using the same sampling technique and sampling point in the manufacturing process as the sample taken for use in the quality control release of the lot.

EXCIPIENTS FOR WHICH PURITY CAN BE MEASURED

A mass balance is desirable, but it is recognized that a mass balance of 100% cannot generally be achieved because of the inherent limitation in accuracy and precision of the available tests, as well as the possible lack of suitable tests for some components. Mass balance of the excipient composition should be computed through the addition of the organic impurities, inorganic impurities, residual solvents, and excipient. If there are measurable essential concomitant components, they should be included with the excipient for purposes of this calculation. The purpose of calculating the mass balance is to estimate the amount of material not measured in the impurity profile. The excipient manufacturer should include in the report of the development of the impurity profile the mass balance achieved and what are thought to be the components not fully quantified.

Organic Impurities: Identify each impurity at or greater than 0.10% using appropriate analytical techniques. If organic impurities cannot be identified, a qualitative description, such as chromatographic retention time, should be assigned for all impurities at or greater than 0.10%. If direct measurement of organic impurities is not possible, total *Organic Impurities* can be reported as:

$$100 - (\text{Inorganic Impurities} + \text{Residual Solvents} + \text{Excipient})$$

Inorganic Impurities: Identify each impurity at or above 0.10% using appropriate analytical techniques. If direct measurement of inorganic impurities is not possible, total *Inorganic Impurities* may be estimated as:

$$100 - (\text{Organic Impurities} + \text{Residual Solvents} + \text{Excipient})$$

Residual Solvents: Report the solvents present by classification (see *Residual Solvents* ⟨467⟩) and level.

EXCIPIENTS FOR WHICH PURITY CANNOT BE MEASURED

Although a mass balance of the excipient composition of 100% is desirable, it is recognized that this goal is often technically difficult, if not impossible, to achieve. Therefore, manufacturers should include reports of the development of the impurity profile, the mass balance achieved, and what are thought to be the components not otherwise quantified.

For excipients produced by continuous chemical processing, it may not be possible to calculate a chemical mass balance, only an overall process balance.

Where direct measurement of the excipient purity is not feasible, techniques should be used to provide an estimate of excipient purity. This information is then applied in the equations listed above under *Excipients for Which Purity Can Be Measured*.

DOCUMENTATION

The excipient supplier should develop documentation to support the development of an impurity profile. This documentation can be compiled in various ways by the supplier so that it can be retrieved to support the impurity profile. Documentation of an excipient impurity profile should include the following information:

1. Sampling plan
2. Analytical test methods
3. Identity and quantity of each component of the excipient, including both the excipient components and identified impurities
4. Discussion of the uncertainty in the measurement of each component of the excipient and impurity
5. Discussion of the mass balance

〈1197〉 GOOD DISTRIBUTION PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

SECTION 1. INTRODUCTION AND SCOPE

1.1 Introduction

Excipients are used in virtually all drug products and are essential to product performance and quality. Typically, excipients are manufactured and supplied so that they comply with compendial standards. The pharmaceutical excipient supply chain participants include manufacturers, distributors, brokers, suppliers, traders, transporters, forwarding agents, and repackagers. The quality of pharmaceutical excipients is affected by inadequate control of activities including distribution, packaging, repackaging, labeling, and storage. Improper or inadequately controlled trade and distribution practices can pose a significant risk to the quality of pharmaceutical excipients and can increase the risk of contamination, cross-contamination, adulteration, mix-ups, degradation, or changes in physical or chemical properties. To maintain the original and intended quality, all participants in the excipient supply chain should carry out their activities according to appropriate standards for good trade and distribution practices as discussed in this chapter.

NOTE: The Appendix consists of definitions and acronyms.

1.2 Scope

This general information chapter provides recommendations for those activities and practices that ensure good trade and distribution practices for pharmaceutical excipients in order to ensure their intended quality. These activities and practices include quality management, organization, documentation, premises, storage, equipment, stability, prevention of adulteration, importation, packaging, repackaging, labeling, dispatch, transport, returned goods, and compounding practices. In addition, personnel, authenticity of data, expiration dating, retesting, complaints and recalls, handling of nonconforming materials, internal/external/third-party audits, quality agreements, shelf life, traceability, economically motivated adulteration, and conformance to compendial monographs are included. The procedures outlined here are applicable to all persons and manufacturers involved in the handling of pharmaceutical excipients and apply to every step in the supply chain. This chapter covers all materials designated as, or intended for use as pharmaceutical excipients, beginning with the point in the manufacturing process at which the final excipient is designated for pharmaceutical use.

1.3 General Considerations

Manufacturers, distributors, users, regulators, and consumers expect pharmaceutical excipients to be manufactured, packed, stored, and transported in a manner that does not compromise their suitability for use in medicinal products for human or veterinary use. Because they are components of drug products, excipients are drugs within the meaning of the U.S. Federal Food, Drug, and Cosmetic Act (FD&C Act), and thus the U.S. Food and Drug Administration (FDA) definition of adulteration applies when an excipient is not fit for its intended use.

Excipients are a diverse group of materials. They can be of animal, mineral, synthetic, or vegetable origin, and they include materials that are solids, liquids, or gases. Excipients can be packed and transported in container sizes ranging from a few grams to a railroad tank car.

Because of their diverse nature and the number of ways in which excipients can be transported from the manufacturing site through the supply chain to the ultimate site of use, this general information chapter cannot provide exhaustive detail for specific materials and modes of transport. Rather, this chapter provides general guidance about what is expected of those people and organizations involved in the supply and distribution of pharmaceutical excipients intended for use in the manufacture of pharmaceutical finished products. Hence, there are instances when *USP–NF* chapters *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩, *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩, and *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩ provide a more detailed guide about what is expected in these specific areas.

Excipients also are used in a variety of industries. Although most drug substances typically are made exclusively for use in pharmaceutical finished products, the pharmaceutical use of an excipient may be only a small fraction of the total use of the material across all industries. This complicates the regulation of both the manufacture and the supply of pharmaceutical excipients. Excipients often are manufactured outside the United States, which further complicates the regulation of the manufacture and the supply of pharmaceutical excipients. Thus, all stages in the supply chain for the pharmaceutical excipient require transparency and proper flow of the necessary information regarding the excipient shipment. In addition, to ensure compliance with this chapter, suppliers of pharmaceutical excipients must follow all applicable national, regional, and local laws and regulations.

1.4 Pharmaceutical-Grade Excipients

Pharmaceutical excipients must be prepared according to the recognized principles of good manufacturing practices (GMPs) using ingredients that comply with specifications designed to ensure that the resulting substances meet the requirements of the compendial monograph (see *General Notices 3.10* and chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

USP or *NF* standards apply to any excipient marketed in the United States that is recognized in the compendium and is intended or labeled for use as an ingredient in a pharmaceutical product. The applicable standard applies to such articles whether or not the added designation *"USP"* or *"NF"* is used (see *General Notices 3.10.10*). An ingredient may include the designation *"USP"* or *"NF"* in conjunction with its official title or elsewhere on the label only when a monograph is provided in the compendium and the article complies with the monograph standards and other applicable standards in the compendium including, but not limited to, the principles of GMP manufacture (see *General Notices 3.20*).

When *USP*- or *NF*-grade excipients are unavailable, manufacturers should first explore the use of materials which claim to comply with other pharmacopeias (e.g. EP, JP). If unavailable, pharmaceutical manufacturers should then consider appropriate alternatives (e.g. food-grade ingredients), provided such materials are suitable for the intended use. If a pharmacopeial grade is not used, a written justification should be available. The pharmaceutical manufacturer/user is responsible for the development and confirmation of suitable quality tests, procedures, and attributes to ensure that the material is appropriate for its intended use and that manufacturing is carried out under GMPs or a quality management system that demonstrates the same level of assurance of quality as that provided in *USP* (see ⟨1078⟩). It is an unacceptable practice to upgrade technical- or industrial-grade material to pharmaceutical-grade quality based only on analytical results that show compliance with the requirements of a pharmacopeial monograph.

1.5 Authenticity of Data

In the United States, the responsibility for the quality of the components of a finished pharmaceutical product lies with the organization that guarantees the quality of the finished pharmaceutical product. Thus, an important consideration in the purchase and supply of a pharmaceutical excipient is confirmation that the material is what it purports to be, that it meets specifications, that it was manufactured under applicable GMPs, that it has not been tampered with in any way before arriving at the site of intended use, that the appearance of the containers and other attributes of the shipment are comparable to those of previously received shipments of the same excipient and grade from the same supplier, and that it is fit for its intended use. Certain paperwork should accompany all shipments of pharmaceutical excipients. This paperwork should include a bona fide and legible copy of a Certificate of Analysis (COA) (see *Bulk Pharmaceutical Excipients—Certificate of Analysis* ⟨1080⟩).

When they receive a COA, manufacturers should take appropriate steps to verify the authenticity of the COA and the data contained therein. This has become particularly important in recent years because of instances of adulteration of excipients

intended for use in the manufacture of pharmaceutical products. Steps to verify the authenticity of the COA should be taken at all stages in the supply chain.

Data on the COA can be verified in a number of ways, but the excipient user is responsible for confirming that the data are authentic by means of periodic verification of compliance with established specifications as stated in 21 Code of Federal Regulations Part 211 (21 CFR 211; see *Current Good Manufacturing Practice For Finished Pharmaceuticals*, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=211, http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM095852.txt). In addition, other documents such as dispatch notes from previous stages in the supply chain can provide further evidence of the pedigree of the excipient shipment. Such documents are termed "pedigree documents".[1]

This chapter may present additional challenges for certain excipient users, e.g., compounding pharmacies. However, those who compound still are obliged to take all reasonable steps to verify that excipients they receive are fit for their intended use. Part of this verification can include an examination of pedigree documents and a signed certificate of conformance (COC) from the suppliers. Information contained in the *USP–NF* monograph's labeling requirements, FDA's Inactive Ingredient Database, and the CFR provide specific information about the excipient's permitted use in FDA-regulated products. All purchasers of pharmaceutical excipients should establish written procedures for the verification of data and verification that the excipient is fit for its intended purpose.

SECTION 2: QUALITY, ORGANIZATION, AND DOCUMENTATION

2.1 Quality Management

A Quality Management System (QMS) is a tool by which all parties involved in the excipient supply chain maintain the quality of the excipient. A documented quality policy is the cornerstone of the QMS and formally describes the company's overall philosophy with regard to quality as authorized by top or senior management. Additionally, an appropriate QMS should include:

- An organizational structure capable of supporting the elements of the quality policy
- Documented procedures and relevant records that demonstrate that a product will meet established quality criteria. This is commonly known as quality assurance (QA)
- Established procedures for approving suppliers of starting materials and verifying that they continue to meet agreed-upon requirements
- A material-release testing procedure to confirm the quality of excipients for their intended purpose(s)

Excipient manufacturers and suppliers should prepare a Quality Manual. The Quality Manual describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes (see *Section 2.3 Documentation Requirements*). In implementing the QMS, companies must ensure that adequate qualified personnel are available to carry out the actions called for in the QMS and must avoid giving any one individual such extensive responsibilities that quality could be at risk.

COC to quality systems such as applicable International Organization for Standardization (ISO) guides or hazard analysis and critical control point (HACCP) analyses are not mandatory but provide assurance that products are produced and handled appropriately. However, certification to these quality systems should not be viewed as a substitute for the information contained in this chapter. In addition, internal audits should be conducted at regular intervals to confirm compliance with GMP (as applicable) and good distribution practices (GDP), and manufacturers should seek opportunities for improvement (see *Section 2.7 Audits: Internal, External, and Third-Party*).

All parties involved in the excipient supply chain share responsibility for the quality and safety of pharmaceutical excipients. These responsibilities should be delineated in a quality agreement between parties in the supply chain (see *Section 2.9 Quality Agreements*). All parties and their activities in the supply chain should be documented, and records should be maintained according to written procedures that ensure the traceability of all products acquired and distributed. All members of the supply chain have an obligation to protect excipients in their custody from deliberate economically motivated adulteration or deliberate introduction of foreign materials that could compromise the quality or performance of the excipient or adversely affect human or animal health.

2.2 Organization and Personnel

The organizational structure should be adequate and sufficiently staffed, and workers should be appropriately authorized for the activities they conduct. An organizational chart should delineate the responsibilities and interrelationships of personnel. Management ultimately is responsible for implementation of GDPs and ongoing verification that the QMS is maintaining the intended excipient quality.

Individuals within the company should have clearly defined responsibilities that are documented in writing. All individuals should understand their responsibilities and should be suitably qualified to perform their assigned duties. Their qualifications

[1] IPEC. The IPEC Excipient Pedigree White Paper. Arlington, VA: IPEC; ND. Available at: http://ipecamericas.org/sites/default/files/Excipient_Pedigree.pdf (Accessed July 6, 2011).

should be assessed for adequacy for their responsibilities and should be documented. Qualifications can include a combination of formal education, training, and experience. This also extends to any contracted service providers. Procedures should be in place to ensure that permanent, temporary, and contract employees minimize the possibility that unauthorized individuals will handle products.

An employee at each supply chain site should be designated and given the authority and responsibility for the implementation and maintenance of the QMS. The designated employee should have sufficient authority, qualifications, and resources to perform this function, as well as to identify and correct deviations from the QMS. Management and other personnel must not be subject to conflicts of interest or other pressures that could have an adverse effect on their ability to perform their duties related to product quality.

Staff should be aware of the principles of GDP included in this chapter and should receive regular, on-going training relevant to their responsibilities and to general quality principles. All training should be conducted according to a written training plan, and records of this training should be maintained. Personnel who have special duties such as handling hazardous materials or supervising activities required by local legislation may require additional training, including specific hazard management. Effectiveness of training should be verified regularly.

Personnel working with open product must understand and maintain good hygiene, health, and sanitation practices. Staff should use appropriate, nonshedding, protective apparel that will protect the product from the sampler as well as the sampler from the hazards of the product. Established procedures should eliminate the potential for product contamination by personal items such as jewelry, food, drink, or tobacco products. Written procedures that address hygiene, health, sanitation, and protective apparel should be in place.

Each supply channel party should have in place disciplinary procedures to address situations when personnel involved in the handling of products are suspected of or are implicated in inappropriate or illegal activities.

Some quality-related duties may be contracted to third parties, persons, or entities outside of the direct employ of the supplier. The delegation of these activities should be documented in a quality agreement or contract with the third party, and the organization should confirm compliance with the principles of GDP by conducting periodic on-site audits of these third parties. Delegation to a third party does not remove the organization's overall responsibilities for these activities.

2.3 Documentation Requirements

2.3.1 GENERAL

Organizations should have in place a system to control documents and data that relate to the requirements of the QMS.

2.3.2 QUALITY MANUAL

Organizations also should maintain a quality manual that describes the QMS, the quality policy, and the company's commitment to applying the appropriate GDP and quality management standards contained in this chapter. This manual should include the scope of the QMS, reference(s) to supporting procedures, and a description of the interaction between quality management processes.

2.3.3 DOCUMENT CONTROL

Procedures for the identification, collection, indexing, filing, storage, withdrawal, archiving, maintenance, and disposition of controlled documents, including documents of external origin that are part of the QMS, should be established and maintained. Procedures used for the handling and distribution of excipients should be documented, implemented, and maintained. In addition, organizations should establish formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is used throughout the operational areas and that previous revisions of documents have been removed or withdrawn.

Designated qualified personnel should review documents and subsequent changes to the documents before the latter are issued to the appropriate areas. Documents that influence product quality should be reviewed and approved by the quality unit. Controlled documents may include a unique identifier, date of issue, and revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. The reasons for changes and the implementation date should be documented.

Electronic documentation should meet the requirements stated above for the document control system. If electronic signatures are used, they should be controlled to provide security equivalent to that given by a hand-written signature. Electronic documents and signatures also may need to satisfy local regulatory requirements.

2.3.4 CONTROL OF RECORDS AND DATA

Procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records and data should be established and maintained. Records and data should be maintained to demonstrate achievement of the required

quality and the effective operation of the QMS. Records and data should be legible and clearly linked with the product or process involved. Pertinent third-party quality data also should be an element of these records.

Entries in records and data should be clear and indelible and should be made directly after the person performs the activity and then should be signed and dated by the person who made the entry. Corrections to entries should be signed and dated, leaving the original entry legible and with an explanation for the change, especially if this may not be obvious to subsequent reviewers.

Records and data should be kept for a defined period that is appropriate for the excipient, its use, and its retest or re-evaluation date. Records and data should be stored and maintained in such a manner that they are readily retrievable and in facilities that provide a suitable environment to minimize deterioration or damage. Electronic records and automated data-capture systems should meet the requirements for controlled records and data as stated above.

2.3.5 CHANGE CONTROL

Procedures to evaluate and approve all changes, including evaluating the impact of the change on the quality of the excipient, should be established and maintained, for example, changes to the following:

- Authorized excipient manufacturer or packaging material supplier
- Manufacturing or packaging sites
- Excipient or packaging material specifications
- Test methods and laboratory
- Repackaging, labeling, and storage equipment
- Analytical equipment
- Repackaging, labeling, and storage processes
- Process and equipment changes at the original excipient manufacturer's site (see *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩)

An independent QA group should have the responsibility and authority for the final approval of any changes. The QA group may be part of another operational unit such as regulatory affairs or research and development.

Customers and, if applicable, regulatory authorities (e.g., those responsible for drug master files or certificates of suitability to the *European Pharmacopoeia*) should be notified of significant changes to established production and process control procedures that could affect excipient quality. The original manufacturer and downstream intermediaries (distributors and traders) should have excipient change control agreements in place defining the extent of notification by the original manufacturer in case of a change as described above. Each of the handling parties within the supply chain should have change control agreements to ensure that changes from the original excipient manufacturer are communicated to the end user. This change control agreement is part of the overall contractual agreements between the parties.

2.4 Complaints and Deviations

Customer complaints and information about possible defects should be systematically documented and investigated based on a written procedure with assigned responsibilities that describes the action that will be taken and includes the criteria on which a decision to recall a product should be based. Investigations should be formally conducted and written up in a timely manner to establish if the complaint is justified, to identify the origin or reason for the complaint (e.g., the repackaging procedure, the original manufacturing process, etc.), to identify root cause(s), to define any initial and follow-up action(s), and the method of communication (e.g., to the customer, original excipient manufacturer, authorities, etc.). Complaint records should be retained and regularly evaluated for trends, frequency, and criticality in order to identify possibly needed corrective or preventive actions.

Investigations should identify whether the reported defect is limited to a single batch of material or if other batches must be investigated. If additional batches are implicated, they should be identified and labeled accordingly (e.g., "under quarantine"). As necessary, appropriate follow-up action, possibly including a recall (as outlined in *Section 2.5 Recalls*), should be taken after investigation and evaluation of the complaint. Confirmed serious problems related to product quality (e.g., faulty manufacturing, packaging, or product deterioration) should be communicated upstream to the manufacturer and downstream to customer(s) in case they received material with the same batch number. A similar process should be implemented for the handling of deviations and product defects not identified by a customer complaint.

2.5 Recalls

Those involved in the excipient supply chain should have a system for recalling promptly and effectively any materials known or suspected to be defective. Entities involved in the supply chain should implement written procedures to manage excipient recall (retrieval) in a timely manner. The procedures should:

- Describe how the process of recall (retrieval) should be managed based on the risk involved
- Describe a decision-making process with defined responsibilities
- Define the functions involved in the process (e.g., QA, sales, logistics, senior management, competent authorities, etc.)

- Define the communication process and documentation to parties within the supply channel as well as to regulatory authorities
- Define the steps needed to retrieve the material

If the original excipient manufacturer does not initiate a recall, it should be informed of the recall. Entities in the supply chain should have written procedures for the organization of any recall activity, and these should be regularly checked and updated. All recalled materials should be stored in a secure, segregated (quarantined) area while their disposition is decided. In the event of serious or potentially life-threatening situations, all customers and competent authorities in all countries to which an excipient potentially was distributed should be promptly informed of any intention to recall the excipient. All records should be readily available to the designated person(s) responsible for recalls. These records should contain sufficient information about materials supplied to customers (including exported materials). At regular intervals, QA groups in supply-chain organizations should evaluate the effectiveness of recall arrangements.

2.6 Handling of Nonconforming Materials

Nonconforming materials should be handled in accordance with a procedure that will prevent their inadvertent introduction or reintroduction into the market. They should be stored separately, either physically separated or under electronic control, to prevent their inadvertent introduction into commerce. Firms that conduct recalls should maintain records covering all activities, including destruction, disposal, return, and reclassification, and should perform an investigation to establish whether any other batches also are affected. They should document the investigation and actions taken to prevent recurrence of the problem. As necessary, firms should take corrective measures. Procedures should exist for the evaluation and subsequent disposition of nonconforming products, and the disposition of the material, including downgrading to other suitable purposes, should be documented. Nonconforming materials should never be blended with materials that comply with specifications.

2.7 Audits: Internal, External, and Third-Party

To verify compliance with the principles of GDP for pharmaceutical excipients, firms in the excipient supply chain should perform regularly scheduled internal audits in accordance with approved procedures. Firms should document audit findings and corrective actions and ensure that they are brought to the attention of responsible management. Accepted corrective actions should be completed in a timely and effective manner and should be conducted by designated, qualified individuals. Qualified individuals may be employees of the company, but they must be sufficiently removed from the function under audit so that their independence is not compromised.

Firms should perform external audits in accordance with approved procedures and schedules to assess the capability of suppliers to meet requirements for a product or service, as specified. A response to a questionnaire may be considered in the auditing process but generally does not take the place of on-site inspections and should not be considered a substitute when an audit is required. Independent auditing organizations can perform third-party audits to determine the level of compliance or conformance to specified standards and regulations (e.g., GMP, GDP, and ISO).

2.8 Contract Activities

Any GDP-related activity that is delegated to another party should be agreed upon in writing in an approved contract with clearly defined responsibilities. The contract should clearly establish which party is responsible for each applicable quality activity. Before entering into an agreement, the contract giver should evaluate the proposed contract acceptor's compliance with GDP as described in this general chapter. The evaluation should include an initial on-site audit of the contract acceptor's premises and quality system, giving special consideration to the prevention of cross-contamination and maintaining traceability. The contract should also include the responsibilities of the contract giver for measures to avoid the entrance of counterfeit or adulterated materials into the distribution chain.

There should be no gaps or unexplained lapses in the application of GDP. The contract acceptor should conduct periodic on-site auditing of contracted distribution activities with regard to the application of GDP by the contract giver. Subcontracting may be permissible under certain conditions, subject to approval by the original contract giver, especially for activities such as sampling, analysis, repacking, and labeling. If subcontracting occurs, the subcontractor should conform to the same GDP standards as the primary contract giver. The subcontractor also should permit an on-site audit by the contract acceptor's quality unit or its designee.[2,3]

General Chapters

[2] WHO. *Good Trade and Distribution Practices for Pharmaceutical Starting Materials.* Geneva; WHO: Technical Report Series, No. 917, 2003, Annex 2. Available at: http://who.int/medicines/areas/quality_safety/quality_assurance/GoodtradeDistributionPracticesTRS917Annex2.pdf?ua=1 (Accessed March 2, 2015).

[3] WHO. *Finished Products: Good Distribution Practices for Pharmaceutical Products.* Geneva; WHO: Technical Report Series 957, 2010, Annex 5. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_957_eng.pdf (Accessed July 7, 2011).

2.9 Quality Agreements

Quality agreements are legally binding and are mutually negotiated between parties involved in the supply chain for pharmaceutical excipients. The quality agreement identifies who is responsible for certain quality activities and how quality issues will be resolved between the parties. Although they are intended to address the parties' quality commitments, quality agreements are not designed to take the place of an audit.

Suppliers should have in place quality agreements between themselves and the parties with whom they do business. Original excipient manufacturers should have quality agreements in place with their direct customers and authorized distributors of their products. Distributors should also have agreements with end users and other parties in the supply chain to whom they supply products. All entities in the supply chain should fully understand which entity is responsible for the GDP-related activities (as outlined in this chapter) at each step in the supply chain.

Quality agreements should address the quality systems requirements, but they are not intended to list every element of the quality system. It is not necessary to reiterate agreement on every point of the quality system when the parties state general agreement on the applicable quality standard. Quality responsibilities included in a quality agreement should be those that may require action by one or both parties to the agreement.

A key element that must be defined in the quality agreement is the communication pathways and timing for quality events. Parties must be clear about their responsibility for notifying the next party in the supply channel and for notifying the applicable regulatory authorities in the case of a significant quality event. Many times a decision about who should notify the regulatory authority is a collaborative effort between the parties. Depending on the issue's impact, the timing of these notifications relative to the time of the incident should be specified within the quality agreement.

Both parties to the agreement are responsible for ensuring that the quality agreement is maintained as an accurate document throughout the life of the business relationship. Revisions to this document may be needed as regulatory requirements change, new products are supplied, or a new material risk arises. The parties should maintain a history of the revisions to the quality agreement.

SECTION 3: PREMISES, STORAGE, REPACKAGING, AND STABILITY

3.1 Buildings and Facilities

Organizations should establish operating procedures for the use of buildings and facilities, including the areas discussed below, and firms should consider protective measures to ensure the security of the grounds (e.g., fencing or perimeter walls).

The buildings and facilities used in the storage and handling of excipients should restrict access to allow entrance only by authorized persons to areas used for the manufacture, packaging, and holding. Organizations should take precautions to prevent unauthorized persons from entering limited-access areas. When the status of excipients requires protection from use (e.g., quarantine), organizations must have clearly marked limited-access controls in place, or they should use validated computerized systems to prevent material distribution before approved release.

Buildings should be of adequate size and capacity to allow the orderly flow of materials, proper storage and handling of materials, and appropriately controlled environmental conditions for the final dispatch of excipients into and out of the premises. Buildings should be maintained in a good state of repair. The construction materials must be easily cleanable and maintained, and buildings and facilities should be designed to prevent cross-contamination, product mix-ups, or the accumulation of filth or contaminating materials, particularly when excipients are exposed to the environment. Adequate storage space must be available for excipients that are highly sensitizing or toxic, and dedicated facilities may be necessary. Adequate procedures should be in place to ensure the cleaning, maintenance, and use of buildings and facilities.

Receiving and dispatch bays should be designed to protect the facilities and excipients during loading and unloading during adverse weather conditions. Incoming bay areas should be designed and equipped to allow containers to be cleaned before storage. A pest-control system should be in place to ensure that materials are protected from infestation by insects, rodents, animals, birds, or other vermin. There should be written procedures defining the adequate holding and storage of excipients, including pest-control processes. The pest-control materials must be safe and must be known not to cause contamination. Approved pesticides, insecticides, and rodenticides should be used and documented. Excipients that may contain contamination must be controlled to prevent cross-contamination in holding areas or the spread of contamination to other areas of the facility.

3.2 Warehousing and Storage

Written procedures should describe the receipt, storage, dispatch, and other handling of excipients, as well as the security measures necessary to prevent theft of materials or the introduction of counterfeit or adulterated materials into the supply chain. Buildings should be adequately lighted and should have proper utilities for the intended activities. They should be dry and controlled to appropriate environmental conditions. Buildings and facilities should store excipients in the proper environmental conditions. Temperature-controlled and -monitored storage should be available as required for any building used for holding excipients.

Warehousing and storage conditions for excipients should comply with the monograph specifications, as reflected in the excipient's container label. When specific storage conditions are required for excipients (e.g., temperature and humidity control), they should be provided in a controlled manner, monitored (e.g., by an alarm system or manual control), and recorded. Any automated system(s) used to monitor the environmental conditions for areas where excipients are handled or stored must be validated. An approved document should indicate the location of each environmental monitoring device and the condition(s) it monitors. The locations for these devices or probes should reflect the extreme environmental conditions of the space as determined by an environmental mapping exercise. Excipients that present risks such as fire or explosion should be stored in safe, dedicated areas. Excipients that are sensitizing or toxic should be adequately and appropriately segregated, and warehouse and storage areas should be routinely cleaned, appropriately maintained, and free of pests.

Excipients should be stored in a manner that permits cleaning of the storage area and movement of materials. Pallets used to hold materials should not cause contamination, and required pallet quality and construction materials should be defined in writing. Pallets should be clean and in a good state of repair, and firms should appropriately track supplies to ensure adequate treatment of the wood materials. Wood pallets, if used, should comply with import requirements.

Organizations should have in place written procedures to ensure that the excipient will be supplied within its expiry or retest period and should have adequate controls to prevent the distribution of expired excipients. If no expiry date is applicable, the first-in-first-out principle should be used. Rejected excipients and other materials related to excipient quality (e.g., packaging components) should be so labeled or identified, and controls such as physical or electronic separation should prevent their use pending final disposition. During the warehousing or storage of excipients, any known broken or damaged containers should be withdrawn from usable stock, and the containers should be handled as rejected materials.

Materials quarantined pending a release decision should be labeled or identified (e.g., electronically) to prevent unauthorized use. These materials should be held from use, and written procedures should guide final disposition. There should be written procedures for the cleanup of any spillage to ensure complete removal of any risk of contamination.

3.2.1 ENVIRONMENTAL CONTROLS

When excipients require specific storage conditions to preserve their integrity and quality during the retest/re-evaluation or expiry interval, the storage conditions required should be stated on the label, labeling, or other literature, e.g., the Excipient Information Package[4] or COA. Distributors should follow the information and requirements for environmental controls provided by the manufacturer and should provide appropriate controls and monitoring to ensure adherence to the stated storage conditions with appropriate documentation. Distributors should also maintain records to indicate the excipient was stored according to the manufacturer's recommendations and should conduct regular assessments to confirm that designated conditions are met.

If the manufacturer does not indicate specific storage conditions, the distributor should ensure that proper storage conditions are maintained to protect the packaging and labeling. Uncontrolled warehousing conditions vary with geographical location, particularly with latitude. If the excipient is shipped to geographical locations that have storage conditions well outside the conditions used in the manufacturer's stability study or justification for the absence of special storage conditions, then additional studies may be required to show stability at the new conditions. A warehouse-monitoring program should be established if the effects of the new environmental conditions are not known.

Outdoor storage of excipients (for example, bulk materials, flammable materials, acids, or other corrosive substances) is acceptable provided the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, discharge ports have adequate protective closures, and the exteriors of moveable containers are adequately cleaned before opening and use.

3.3 Equipment

Equipment used in bulk transport, repackaging, labeling, testing, or storage of the excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation. Equipment should be verified before use to ensure that it is constructed, installed, and functioning as required for the excipient. When equipment is located outdoors, there should be suitable controls (e.g., closed systems or protective encasements) to minimize environmental risks to excipient quality.

When possible, dedicated equipment (e.g., bulk trucks, packaging equipment, storage tanks, pipework, hoses, and pumps) should be used in direct contact with the excipient. When nondedicated equipment is used in direct contact with the excipient, validated cleaning procedures should be applied. A restricted prior-cargo list should be supplied to transport companies in case non-dedicated bulk transport equipment is used. Quality-critical measuring equipment and balances for the handling and testing of the excipient should be of appropriate range and precision. Such equipment should be identified.

[4] IPEC. *The IPEC Excipient Information Package (EIP): Template and User Guide.* Arlington, VA: IPEC; 2011. http://ipecamericas.org/ipec-store.

General Chapters

3.3.1 EQUIPMENT CONSTRUCTION

Equipment in contact with an excipient should be constructed so that contact surfaces are not reactive, additive, or absorptive and thus do not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, preferably should not come into contact with excipients and packaging materials. When contact is possible, distributors should use materials of suitable quality that will not affect product quality. The choice of such materials should be justified.

Equipment should be designed to minimize the possibility of contamination from the environment and direct operator contact during activities such as unloading bulk trucks, use of transfer hoses (particularly those used for transfer of excipients), sampling, repackaging, and cleaning. Distributors should consider the sanitary design of equipment in contact with excipients. They should assess the suitability and integrity of seals in order to minimize the risk of contamination. Piping should be appropriately labeled to indicate the content and direction of flow.

3.3.2 EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the repackaging, labeling, testing, or storage of the excipient. Distributors should maintain records (e.g., logs, computer databases, or other appropriate documentation) of quality-critical equipment use and maintenance. Defective equipment should be removed or appropriately labeled to avoid misuse.

Quality-critical measuring equipment and balances should be controlled on a scheduled basis. This control should include the following:

- Calibration of instruments or other appropriate verification at suitable intervals, according to an established documented program
- Establishment of the equipment's limits of accuracy and precision
- Provisions for remedial action in the event that accuracy or precision requirements are not met

Calibration standards should be traceable to recognized national or compendial standards as appropriate. Instruments and equipment that do not meet established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration or verification status of quality-critical equipment should be known to users and should be verifiable.

3.3.3 EQUIPMENT CLEANING

Cleaning equipment should be chosen and used so that it cannot be a source of contamination. Cleaning materials should be appropriate for the task, and their selection should be justified. Rotation of sanitizing and cleaning agents should be considered where appropriate. In order to avoid contamination with cleaning products or products previously processed in the equipment, written cleaning procedures should be established for equipment that comes in contact with the excipient. Cleaning procedures should contain sufficient detail to allow cleaning in a reproducible and effective manner. Cleaning and sanitation processes should be recorded, and evidence of their effectiveness should be provided, for example, by the following:

- Testing the final rinse after cleaning for residues of the previous product
- Checking the equipment after cleaning for residues of the previous product
- Testing each batch for residues of the previous product handled with the same equipment

3.4 Sampling, Repackaging, and Labeling

To minimize risks associated with repackaging and labeling, appropriate GMPs should be applied (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩). For completeness, certain key activities and the necessary precautions are discussed below.

3.4.1 BLENDING, REPACKAGING, AND LABELING

Operations such as combining sublots into a homogeneous batch, repackaging, or labeling are manufacturing processes, and therefore distributors should follow appropriate GMPs (see ⟨1078⟩):

- Processes whereby the excipient's packaging is opened and the excipient is exposed to the environment (for example, transferring excipient from one container to another, including from bulk equipment to storage tanks/silos or from storage tanks/silos into containers) are critical handling steps related to the integrity of the finished product. If only the secondary packaging is modified, operators should take appropriate care to maintain the integrity of the primary packaging and the excipient.
- Excipients may degrade because of exposure to the repackaging atmosphere (e.g., oxygen, humidity, light, and temperature).
- Excipients can be contaminated by foreign matter such as lubricants, cleaning materials, or other substances.

- Transparency to the customer that relabeling, with or without opening the original excipient manufacturer's packaging, has occurred is critical to representation of the product quality and suitability for use.
- Transparency to the customer of data sources listed on certification documentation (labeling) is critical to representation of the product quality and suitability for use.

3.4.2 REPACKAGING AND LABELING BATCHES

Staff in the excipient supply chain should give special attention to the following points:

- All repackaging and labeling requirements should be defined in written procedures.
- Contamination, cross-contamination, and mix-ups should be avoided by the use of suitable equipment and cleaning procedures and with adequate labeling.
- Environmental conditions and repackaging procedures should be designed to avoid contamination and to maintain the integrity of the excipient during repackaging and labeling.
- Operators should consider the use of filtered air in the repackaging area if necessary for the product. The standard of filtration should be justified.
- Labels should be printed using a controlled process (see *Section 3.4.9 Repackaging and Labeling*).
- Personnel involved in repackaging processes should wear clean protective apparel such as head, face, hand, and arm coverings, as necessary, and should practice appropriate personal hygiene (e.g., hand disinfection following health requirements, health monitoring, and removal of jewelry). Personnel should be trained about special hygiene requirements, and this training should be documented.
- Repackaging areas should be cleaned and sanitized regularly.

Batch numbers should be assigned according to documented procedures. When staff assigns new batch numbers, they should ensure traceability to original batch numbers by proper documentation. Assigning one batch number to containers of different batches that comply with the same specification is an unacceptable practice (see also *Sections 3.4.3 Excipient Batch Homogeneity* and *3.4.4 Blended Excipients*).

- As part of the batch record, a copy of the information on the original labels should be retained (e.g., a photocopy). A sample of the new label should also be kept.
- All repackaging and labeling processes should be designed and carried out to avoid commingling, contamination, and mix-up and to ensure full traceability of the excipients back to the original excipient manufacturer and traceability downstream to the final customer. Responsible personnel should sufficiently record every completed step, along with the name of the operator and the date and time each step was completed, e.g., in the master batch manufacturing record, or by means of computerized systems.

3.4.3 EXCIPIENT BATCH HOMOGENEITY

Mixing to form a homogeneous batch is a manufacturing step and should be defined in a written procedure. A batch can be homogeneous only when conforming materials are thoroughly mixed. The conformity of each batch with its specification should be confirmed before it is added. Mixing should always be controlled, and homogeneity should be verified and documented (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩). Blending of batches or lots of excipients that individually do not conform to specifications with other lots that do conform (in an attempt to salvage or hide adulterated or expired material) is not an acceptable practice. Only excipients from the same manufacturing site received by a distributor and shown to conform to the same specifications can be mixed. The customer should be informed that the material supplied is a mixture of the manufacturer's batches.

3.4.4 BLENDED EXCIPIENTS

The blending process should be verified to ensure that it does not influence the quality of the excipient. The blended excipient should be tested to ensure conformance to the specification and to provide data for the COA (see *Significant Change Guide for Bulk Pharmaceutical Excipients* ⟨1195⟩). Under certain circumstances and with appropriate controls, a COC can be used if the basis for the claim of conformity is traceable within the document. The blended batch referred to in the new certification document should be traceable to all the original certification documents and batch numbers (see ⟨1078⟩).

3.4.5 CERTIFICATES OF ANALYSIS

The original excipient manufacturer's COA should be retained and made available to the user on request. The batch referred to in the COA delivered to the end user should be traceable to the original excipient manufacturer's COA. Quality documents accompanying deliveries should be subject to an agreement between the distributor and the final customer. For retesting, analytical methods of the original excipient manufacturer or pharmacopeial methods should be applied. When other methods are applied, these should be agreed upon by both parties.

3.4.6 CONTAINER–CLOSURE SYSTEMS

For repackaged material, the repackager is responsible for justifying the shelf life and repackaging conditions. The original manufacturer and the distributer should share information and agree about repackaging conditions and primary packaging materials. They should establish primary container–closure system material and packaging configuration specifications, and they should develop a written procedure that clearly defines packaging for each individual excipient based on its stability.

If the same types of primary container–closure system and packaging configuration are used for repackaging, then the new container–closure system and packaging configuration should be equivalent to that used by the original excipient manufacturer. The repackager and distributor should consider exposure of the excipient to the repackaging environment, and both can rely on the manufacturer's stability evaluation and thus assign the same shelf life for the excipient.

When the repackager's primary container–closure system's packaging configuration differs significantly from that of the original manufacturer [e.g., in terms of desiccants, permeability of the protective barrier layer (which may be either the primary or secondary container–closure system), or the headspace], the repackager must demonstrate that the new system is adequate to protect the excipient from contamination and deterioration for the shelf life (retest or expiration period) defined by the excipient manufacturer. Otherwise, the shelf life defined by the manufacturer cannot be transferred to the repackaged material. The need for stability studies should be confirmed (see *Sections 3.4.14 Stability and Expiration Dates* and *3.5 Retesting and Shelf Life*).

The container–closure system for the pharmaceutical excipient should protect the material from the time of packaging until its final use by the drug product manufacturer. The container–closure system should be designed to help prevent theft or adulteration by counterfeiting.

Storage and handling procedures should protect containers and closures and minimize the risk of contamination, damage or deterioration, and mix-ups (e.g., between containers that have different specifications but are similar in appearance).

3.4.7 RETURNED AND REUSED CONTAINERS

Returned containers may have unknown residues from uses other than the intended one. Therefore, use of new containers is recommended for excipients. If containers are reused, a rationale for the extent of cleaning should be justified and documented for specific excipients and different types of containers. Repackagers should collect evidence that the quality of the material packed is not adversely affected by reuse of containers.

Distributors and customers should have an agreement defining the specific conditions for reuse (e.g., handling, sealing, and cleaning). If returnable excipient containers are reused, all previous labeling should be removed or obliterated.

3.4.8 ENVIRONMENTAL CONTROLS

Environmental controls should ensure that temperature, humidity, and cleanliness of air and equipment are appropriate to avoid any contamination to or deterioration of the excipient. The necessary environmental conditions for the repackaging of each excipient should be defined. Environmental control is a specialist subject, and experts should be consulted (see also *Section 2.6 Handling of Nonconforming Materials*).

3.4.9 REPACKAGING AND LABELING

Repackagers should implement procedures to ensure that the correct quantity of labels is printed and issued and that labels contain the necessary information. Sufficient crosschecks should be in place to ensure proper data transfer. Procedures should be in place to avoid mislabeling, and printing and use of labels should be restricted. All labeling operations (e.g., generating, printing, storage, use, and destruction) should be recorded. Labeled containers should be inspected, and surplus labels should be destroyed to avoid any misuse. If labels are not printed immediately before each specific labeling operation, the security of the label stock should be controlled, and access limitations should be defined. Repackaging and labeling facilities should be inspected immediately before use to ensure that all materials that are not required for the next repackaging operation have been removed.

3.4.10 REPACKAGED EXCIPIENTS—ACCOMPANYING DOCUMENTATION

Deliveries of repackaged excipients should be accompanied by information about the original manufacturing site (name and address) and repackaging and labeling sites. This information should be provided in the supplier certification documentation (e.g., COAs) or by other means (see *Section 4.8 Traceability*). The supplier should provide this information to the customer via official communications.

General Chapters

3.4.11 TESTING OF REPACKAGED EXCIPIENTS

Appropriate testing of repackaged excipients should be performed to demonstrate consistent excipient quality. Testing to the complete monograph may not be necessary, but the recipient should test defined key quality parameters that could be affected by the repackaging process. Recipients should consider the manufacturer's recommendations for key quality parameters, and until these tests have been performed the repackaged materials should be kept under quarantine and should be identified as quarantined material. The materials should comply with the defined specifications before they are released for distribution.

Excipient testing and release should be performed under the responsibility of the quality unit and should conform to written specifications and analytical test requirements. Repackagers should ensure that test data are recorded and that results are evaluated before release of the repackaged or transferred excipient.

The excipient cannot be upgraded as a result of any repackaging process. It is unacceptable to upgrade nonpharmaceutical grades to pharmaceutical grades on the basis of conforming analytical results, i.e., by testing to pharmacopeial standards. Pharmaceutical grades can be achieved only when the excipient is originally produced and subsequently processed in accordance with GMPs (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* ⟨1078⟩).

3.4.12 OFFICIAL PHARMACOPEIAL METHODS FOR RETESTING

For control of key parameters during repackaging or full retesting of excipients, official pharmacopeial methods or methods validated against the pharmacopeial methods should be used. Otherwise, repackagers should use the original excipient manufacturer's analytical methods. The methods used should be listed on the COA accompanying the excipient or should be made available to the customer by other documents. These documents should also reference any contract laboratory that is used to perform analyses. The COA should clearly identify which tests have not been performed on the repackaged or transferred batch but have been taken from the original manufacturer's COA.

3.4.13 SAMPLING

Excipient sampling must be done in a manner that prevents contamination, and dedicated sampling areas with adequate environmental controls are necessary. Areas for sampling should be designed to allow cleaning of the outside of the container before the container is opened. Adequate cleaning procedures should be in place for the sampling areas. Sampling tools should be dedicated to the sampling area and also to the specific material, or sampling tool cleaning must be validated to ensure no cross-contamination from the tool.

Any container opened for sampling should be marked with the date and name of the person who performs this operation. The amount of sample removed should be recorded.

If excipients are repackaged, processed, or packaged from bulk, retained samples representative of the excipient batch should be kept for at least one year after the expiration or re-evaluation date or for at least one year after distribution is complete, whichever is longer. The minimum sample size should be based on the amount required to perform at least two complete analyses. Sample storage conditions should prevent any contamination or deterioration and should comply with the label storage conditions (see general information chapter, *Bulk Powder Sampling Procedures* ⟨1097⟩).

3.4.14 STABILITY AND EXPIRATION DATES

Excipient stability and expiration dating of excipients are primarily the responsibility of the original manufacturer. Whenever the original manufacturer's packaging is opened, the repacker is responsible for providing evidence that the excipient manufacturer's stability and expiration dating are still applicable.

If a distributor transfers an excipient to another container or repackages it, stability and shelf life (retest or expiry period) should be taken into account. The type of container, primary packaging materials, barrier packaging materials, packaging configuration, environmental exposure during repackaging, and storage conditions at the repackaging site should also be taken into account when the shelf life (retest or expiry period) is defined. The recommended expiration date provided by the original excipient manufacturer should not be extended without demonstrating sufficient stability to justify extended shelf life (retest or expiry period). If shelf life is extended beyond the original manufacturer's recommendation, the type of packaging, storage conditions, and stability-indicating analytical data should be clearly defined, and the repacker assumes the primary responsibility for the extension.

If special storage conditions (e.g., inert gas overlay, protection from light, heat, moisture, etc.) are needed, the restrictions should be indicated on the new labeling (see *Section 3.5 Retesting and Shelf Life*).

3.5 Retesting and Shelf Life

The organization's stated shelf life or retest/re-evaluation interval should be maintained for the excipient. Expiration or shelf life dates indicate the period beyond which the excipient should not be used or distributed. Retest/re-evaluation intervals indi-

cate the period beyond which the excipient must be evaluated to determine continuing acceptability for use. The expiration/ shelf life date provided by the original excipient manufacturer should not be extended without documentation from the manufacturer demonstrating sufficient stability to justify an extended shelf life. Such documentation should specify the type of container and storage conditions necessary to make this claim, and the distributor should have documentation that the excipient was stored in the stated container and under the necessary conditions.

Excipients without expiration, retest, re-evaluation, or shelf life dates should be accepted for use only if the manufacturing date can be confirmed and only if the excipient has been held and shipped under conditions that conform to the appropriate standards of GMP or GDP. Distribution of the excipient beyond the retest/re-evaluation period should be done only in consultation with the manufacturer and with the consent of the purchaser or recipient. If the distributor has the capabilities for sampling and performing the manufacturer's specified evaluation, then the distributor can perform the assessment. Sampled lots should be placed under quarantine to prevent shipping during the evaluation.

Distributors who do have capabilities for sampling according to the manufacturer's instructions but do not have testing or evaluation capabilities should send the samples to the manufacturer or a qualified third-party laboratory for retesting/re-evaluation. Excipient lots that conform to the manufacturer's criteria can be released from quarantine, and the distributor's supporting evaluation data should accompany the original excipient manufacturer's data to indicate the excipient's acceptability for use. If the distributor does not have the capability to sample or evaluate the excipient, it should not be shipped to customers beyond the end of the retest/re-evaluation interval. The excipient or a representative sample of the excipient can be returned to the manufacturer or a third party for retesting/re-evaluation. The excipient can be held by the distributor pending further results obtained from the representative sample.

If an excipient is transferred to another container or is repackaged by the distributor, the latter must conduct an assessment of the stability of the excipient to determine if the original excipient manufacturer's information can be carried forward. If the distributor uses the same type of packaging material that provides the same packaged environment (headspace, surface area, closure tightness, etc.) as that used by the original manufacturer and if the transfer or repackaging is performed in a manner that protects the excipient from adverse environmental effects that could affect the stability, then the original excipient manufacturer's shelf life/expiry date or retest/re-evaluation interval can be carried forward. If primary packaging material or barrier packaging material differs from the original excipient manufacturer's primary packaging material or if the packaged environment varies significantly, then an evaluation of the container and its closure system should demonstrate that it is adequate to protect the excipient from deterioration and contamination during the manufacturer's shelf life/expiry date or retest/re-evaluation interval. Otherwise, a stability assessment is necessary to determine the appropriate shelf life/expiry date or retest/re-evaluation interval for the repackaged excipient. Such assessments should be conducted according to the manufacturer's specifications and test methods.

3.6 Expiration Dates

Not all excipients have an expiration date, but if one is assigned it should be displayed on the container and should show the period during which the excipient is expected to remain within specifications if stored properly and after which it should not be used. It is established for every batch by adding the shelf life to the date manufacturing began. The expiration date is based on the type of container and storage conditions, so these parameters should be clearly defined. If special storage conditions are needed (e.g., protection from light, oxygen, heat, humidity, etc.), they should be indicated on the labeling because they could influence usability through the expiration date.[5]

The expiration dates for excipients should be established by documented stability tests or long-term stability data (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩). Occasionally, the expiration date may be established by reference to historical data. Stability involves not only the compendial requirements but also changes in performance properties. Excipient stability tests should determine whether possible degradation, changes in molecular weight and distribution, moisture gain or loss, viscosity changes, microbiological contamination, or other possible changes in excipients could occur when the excipient is stored in a specific container–closure type at specific storage conditions. Stability for repackaged excipients can be found under *Section 3.5. Retesting and Shelf Life.*

3.7 Labels, Icons, and Labeling

3.7.1 LABELS AND ICONS

Label-generating systems and processes should be secure, controlled, and documented. Appropriate verification records should be maintained, and each container should be appropriately identified and labeled. Labels applied to individual small containers should be clear, unambiguous, and permanently fixed in the company's established format. The information on the label should be indelible. Alternative methods can be used for bulk containers/transport and should be justified.

The label may include wording or depict icons to highlight storage and transportation handling requirements and hazards (e.g., avoid dropping, maintain specified environmental conditions, etc.). The use of symbols that are recognized by international organizations is recommended (see *Good Storage and Distribution Practices for Drug Products* ⟨1079⟩). During interna-

[5] IPEC. *The IPEC Excipient Stability Program Guide 2010.* Arlington, VA: IPEC; 2010. Available at: http://ipecamericas.org/ipec-store (Accessed July 12, 2011).

tional distribution, the proper language(s) should be used to ensure that handlers understand the requirements set forth on the label.

3.7.2 LABELING

The labeling (which includes both the label and any accompanying documents) should include at least the following information:

- Name of the excipient, including grade and reference to pharmacopeia, as relevant
- If applicable, the International Nonproprietary Name
- Amount (weight or volume)
- Batch number assigned by the original excipient manufacturer or the batch number assigned by the repacker if the material has been repacked and relabeled
- Retest date or expiry date (as applicable)
- Any specified storage conditions, as applicable
- Handling precautions, where necessary
- Identification of the original manufacturing site as agreed with the pharmaceutical customer (see *Section 4.8 Traceability*)
- Name and contact details of the suppliers

SECTION 4: RETURNED GOODS, DISPATCH, TRANSPORT, IMPORTATION, ADULTERATION, AND TRACEABILITY

4.1 Returned Goods

4.1.1 GENERAL

Return of goods by users to suppliers should be reviewed on a case-by-case basis. The distributor should facilitate a root cause analysis and investigation of complaints.

NOTE: Users should document the reason(s) for return of goods to the supplier.

Before returning the goods, if the user identifies and confirms unacceptable product quality the user should provide the supplier with the user's supporting documentation, such as tests and investigation results. If requested, the user should also provide product samples used for tests and investigations. The supplier should be provided an opportunity to conduct thorough investigations to confirm the validity of the user's quality complaint. While the investigation proceeds, the user should quarantine the material in accordance with internal standard operating procedures and should store the material in an area specifically designated for returns, with limited access to operations, and well-separated from incoming or released raw materials.

Goods returned by the user because of excess inventory or other causes unrelated to quality can re-enter commerce within the specified shelf life, provided conditions of storage, transportation, and container integrity have been thoroughly reviewed by the supplier and the quality of the excipient has not been compromised in any way. A formal documented review of each returned container and container tamper-evidence device should be done to verify that these match the container configuration when the materials left the supplier's facility.

If the user opens a commercial packaging container for sampling or investigation (related or unrelated to quality issues) and whether any material was taken out or not, each container should be clearly labeled *Opened.* Written documentation should be provided to the supplier confirming that the container(s) were opened and resealed according to GMPs and describing the reasons for opening, amount withdrawn, and how the pack/container was resealed. Documentation of returned goods should contain a detailed description of all such events including repackaging. Returned excipient containers opened by the user should be clearly identified as such and should not be released as pharmaceutical excipients. In exceptional cases, the material can be released as excipient-grade product if a documented thorough investigation shows no risk of product contamination or deterioration. The quality department should release this material.

Users and suppliers should maintain records of all returned goods, including the product name (trade name and chemical name), batch or lot number, reason for the return, quantity returned, and investigation documentation when applicable. In addition, the supplier should record the final disposition of the material. If returned excipients have been held, stored, or shipped under conditions that could compromise product quality (including ingredients, containers, or labeling), the manufacturer should destroy the excipients. Exceptionally, manufacturers can release the excipients if their examination, testing, and investigations prove that the material meets suitable standards of identity, quality, and purity and that GMPs and GDPs have not been compromised.

4.1.2 DISPOSITION OF RETURNED GOODS

The excipient manufacturer's and/or supplier's quality unit should assess the returned product. The options are the following:

- Return to commerce

- Reprocess
- Regrade to a less stringent standard such as technical or industrial grade (non-GMP use)
- Destroy

Only containers that have not been opened should be considered for return to commerce without further action.

If the quality assessment of returned goods leads to their final destruction and if associated batches are potentially implicated, an appropriate investigation should be conducted and documented to show that the quality of the associated batches is not affected.

4.1.3 REPROCESSING

Reprocessing is a manufacturing step, and the requirements of *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* 〈1078〉 apply. The requirements of 〈1078〉 apply only to those intermediate supply chain entities that undertake reprocessing.

4.2 Shipping and Transportation

4.2.1 SHIPPING

The supplier (the manufacturer or distributor) of pharmaceutical excipients should ensure that the integrity of the pharmaceutical excipient is maintained by the appropriate storage and transport conditions as described in product labeling. After training, staff should follow written procedures for shipping pharmaceutical excipients. These procedures include the requirement to follow the recommended storage and transportation requirements including temperature, humidity, or other special handling precautions.

Actions should be documented when they are performed. Shipping records for pharmaceutical excipients should provide for the following information:

- Date of shipment
- Name and address of the entity that accepted the materials for the transportation
- Mode of transportation
- Name, address, and status of the consignee
- Material name
- Quantity shipped
- Batch number and expiry date
- Required storage and transport conditions (refrigeration, freezing, or controlled room temperature required)
- Shipping code or identification number of the delivery order

When regulatory actions such as FDA Field Alerts or drug product recalls occur, the excipient handler must be prepared to act promptly. Shipping documentation must be sufficient to allow adequate handling of any excipient associated with regulatory action. When reasonable, the shipping schedule for excipients should be documented, and responsibilities can be enumerated in a quality or collaborative agreement between entities to show ownership in the supply chain (Entity A to Entity B; Entity B to Entity C; etc.—see *Section 4.4 Packaging: Tamper-Evident Seals*). The buildings and facilities used to ship materials should be appropriate for their intended use in the storage and handling of excipients (see *Section 3.1 Buildings and Facilities*).

Before loading materials, shippers should inspect the container and vehicle to ensure cleanliness and other consignments (if the shipment is a part load) to ensure no form of contamination is likely to occur. This inspection should be documented according to a written procedure. Materials should not be offloaded into other containers or vehicles without the written permission of the material owner or consignee.

4.2.2 TRANSPORTATION

Materials should be transported in a manner that will ensure the maintenance of controlled conditions as specified by the manufacturer. The transport process should not adversely affect the materials or integrity of the packaging. The supplier of transport services must be provided with the required information in order to maintain specified conditions.

The pharmaceutical excipient manufacturer or supplier should agree with the purchaser for arranging transportation. The need for temperature-controlled storage and transport should be determined using a risk-based approach, taking into account the nature of the excipient, results of stability assessments available from the excipient manufacturer or repackager, the supply chain, and the potential risks to the excipient. If temperature-controlled transportation is contracted, the shipper must have a mechanism for noting and reporting temperature excursions. Labeling on containers and transportation documents should detail the environmental conditions in a manner that provides the transporter or receiver with knowledge and immediate identification of these conditions, if required. The responsibility for ensuring that the proper storage conditions are met rests with each entity that handles, stores, or transports the materials.

Pharmaceutical excipients should be stored and transported in such a way that the identity and integrity of the material are retained, the material does not contaminate and is not contaminated by other materials, and adequate precautions are taken

against spillage, breakage, misappropriation, and theft. The required storage conditions for pharmaceutical excipients should be maintained within acceptable limits during transportation.

Excipients that are potentially dangerous because of the risk of fire or explosion (e.g., combustible liquids, solids, and pressurized gases) should be stored and transported in safe, dedicated, and secure areas, containers, and vehicles. In addition, applicable international agreements and federal regulations should be followed.

4.3 Tampering or Damaged Materials

Materials that are suspected of being tampered with or damaged must be quarantined immediately, and the manufacturer or distributor should be notified. The disposition of the quarantined material should be determined by the excipient manufacturer or supplier, after consultation with the customer. The excipient may be returned to the manufacturer or supplier. Alternatively, arrangements could be made for local certified destruction of the quarantined material. The supplier should make every effort to prevent these materials from being used until an investigation is completed and the final disposition of the material is determined. Written procedures should guide treatment of excipients that have been tampered with or the identification and handling of damaged material.

4.4 Packaging: Tamper-Evident Seals

A tamper-evident package has one or more indicators or barriers to entry that, if breached or missing, can reasonably be expected to provide visible evidence that tampering has occurred. To reduce the likelihood of successful tampering and to increase the likelihood that any breach will be discovered, the package should be distinctive by design or should employ one or more indicators of or barriers to entry. The term *distinctive by design* means that the packaging cannot be duplicated with commonly available materials or by commonly available processes.

A tamper-evident package may involve an immediate container–closure system in direct contact with the contents (primary packaging), a secondary container–closure system not in direct contact with the contents (secondary packaging), or any combination of systems intended to provide visual evidence of package integrity. For primary packaging in direct contact with the excipient (e.g., paper bags), any leak or break should be considered tampering even if the leak or tear is simply accidental damage. For excipients shipped in bulk, using, e.g., tank cars or containers, other means may be appropriate. However, whatever methods are adopted should provide adequate assurance as to the integrity of the excipient being shipped.

Visual examination of the packaging at each stage in the supply chain should provide evidence of repackaging or tampering with commercial packaging. In addition, the manufacturer's name and address, net weight of the material, material name, batch or packaging number, date of manufacture, and date of retest should be identified on a packaging label. The label should be prominently placed on the package and should be unaffected if the tamper-evident feature of the package is breached or missing.

The tamper-evident feature for excipient packaging should be designed so that it remains intact when handled in a reasonable manner from the time of packaging at the site of manufacture and throughout the supply chain—including but not limited to warehouse storage during various phases of the supply chain, transport, distribution, receipt, and storage at the user's facility until use for drug product manufacture.

The manufacturer should communicate tamper-evident features to the downstream members of the supply chain. If the latter observe any evidence that the tamper-evident feature or other part of the package has been compromised in any way, they should quarantine the material immediately and inform the supplier. Appropriate arrangements should be made with the supplier to return the material promptly with a description of the packaging breach. Alternatively, arrangements can be made by the excipient manufacturer or supplier for the local certified destruction of the quarantined material. The user should ensure adequate protection of the breached packaging during shipment to the supplier and can send photographs of the breached packaging to aid the supplier's investigation.

The supplier is responsible for the integrity of packaging, including but not limited to its tamper-evident features, until ownership of the commercial packages is transferred to the user. Material returned because of breach in packaging should be thoroughly reviewed and investigated by the supplier. The material should not be returned to commerce until the supplier has established that the integrity, identity, quality, purity, and safety of the excipient have not been compromised. The documentation requirement should comply with GMP expectations as well as elements of documentation and investigation suggested in *Section 4.1 Returned Goods*.

4.5 Where Ownership Begins

The excipient user is responsible for purchased materials throughout the supply chain. The supply chain qualification is documented by audits and COAs for all parties involved in trade and distribution of the materials. Such supply chain qualification and documentation supports the Excipient Pedigree and ownership of the excipient. The pedigree includes documentation of suitable excipient GMPs applied by the excipient manufacturer and suitable GDPs.

Ownership of the materials begins with the original excipient manufacturer and transfers to an intermediary or customer according to agreed-upon terms for insurance costs, transportation, and risk assumption. Such agreements are defined accord-

ing to International Chamber of Commerce terms (Incoterms).[6] Incoterms are a series of international sales terms that are used to divide transaction costs and responsibilities between buyer and seller and reflect state-of-the-art transportation practices.

4.6 Adulteration

4.6.1 ADULTERATION

Adulteration is defined in the FD&C Act and 21 CFR in Sections 501(a)(2)(B) and 501(b)[7] and 21 CFR 211 for finished pharmaceuticals and Sections 402(a)(3) and (4)[8] and 21 CFR 110 for human food, and in 21 CFR 111 for dietary supplements. These laws and regulations establish the minimum current GMP (cGMP) necessary to prevent adulteration for finished pharmaceuticals, food products, and dietary supplements, respectively. Excipients for pharmaceutical use must be manufactured under appropriate GMPs and must meet the required chemical and physical specifications. In addition to specifications, excipient manufacturers and users have generally agreed quality attributes and limits defined by regulatory agencies, common industry practices, and pharmacopeial expectations. Adulteration or contamination of the products can be monitored and detected by many means including, but not limited to, compliance with these predefined quality expectations.

Adulteration can occur when any possible contamination of a product takes place, e.g., from foreign materials or undesirable microorganisms. The problem of adulteration can be addressed by standard practices supporting cGMPs, such as HACCP, Standard Operating Procedures, and staff training to control product safety and purity. This type of adulteration is the unforeseeable and unintentional type that can be controlled and, at worst, detected before the product leaves the manufacturer's site.

FDA specifies that a product can be considered adulterated when conditions *may* lead to adulteration because it is impossible to test every product for every conceivable contaminant. The safety and purity of substances require that manufacturers should build quality controls into the process rather than relying on QC testing.

4.6.2 INTENTIONAL ADULTERATION

Compared to unintentional adulteration, intentional adulteration is more specific because it requires a willful and knowing violation of regulations and standards designed to protect end user safety. It is the deliberate adulteration of an excipient. When an excipient is deliberately adulterated by substituting a lower-cost material for a material of higher cost, it is considered economically motivated intentional adulteration. Every participant in the supply chain should know and monitor their supply chain for any adulterated materials and take all reasonable precautions to prevent intentional adulteration.[9]

4.7 Importation

Excipients manufactured outside the United States are subject to US FDA and US Customs and Border Protection (CBP) regulations for importation into the United States. The Bioterrorism Act (Public Health Security and Bioterrorism Preparedness and Response Act of 2002, Title III—Protecting Safety and Security of Food and Drug Supply)[10] has further formalized the requirements for importation of foods and drugs into the United States.

Overseas manufacturers of excipients used in drugs, food, and dietary supplements intended for human or veterinary use who intend to export products into the United States are required to follow the FDA, CBP, and Bioterrorism Act regulations. A streamlined process for the importation of excipients used in pharmaceuticals, food, and dietary supplements must be followed and implemented before the imports are allowed into the United States.

The manufacturing facilities and the manufacturers who produce the excipients should be registered with FDA. An FDA registration number is required for importation. In addition, FDA requires information about Prior Notice (PN) of Imports. Upon receipt of the information, FDA grants a PN confirmation number. FDA must confirm PN before the products are shipped, and the PN confirmation number must appear on the customs declaration that accompanies the package. PN information can be submitted electronically to FDA's Prior Notice Systems Interface (PNSI), a free Internet application that allows facilities to provide information regarding the expected imports. PN information also can be submitted via CBP's Automated Commercial System (ACS), a system that processes imports and obtains information needed to make decisions regarding articles entering the United States.

CBP processes imports of all goods for entry into United States, including but not limited to pharmaceuticals, food, and dietary supplements. CBP inspects but does not release products regulated by the FD&C Act and delegates the final release re-

[6] International Chamber of Commerce. Incoterms. http://www.iccwbo.org/incoterms (Accessed June 6, 2011).
[7] FD&C Act. Chapter V: Drugs and Devices. Sec. 501. [21 USC §351] Adulterated Drugs and Devices. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterVDrugsandDevices/ucm108055.htm.
[8] FD&C Act. Chapter IV: Food. Sec. 402. [21 USC §342] Adulterated Food. http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/FDCActChapterIVFood/ucm107527.htm (Accessed June 6, 2011).
[9] FDA. Public Meeting on Economically Motivated Adulteration. 2009. http://www.fda.gov/NewsEvents/MeetingsConferencesWorkshops/ucm163619.htm (Accessed June 6, 2011).
[10] FDA. *Guidance for Industry: Questions and Answers Regarding the Interim Final Rule on Prior Notice of Imported Food, (Edition 2); Availability*. 2004. http://www.gpo.gov/fdsys/pkg/FR-2004-05-03/pdf/04-10023.pdf (Accessed June 6, 2011).

sponsibility at the port of entry to the FDA for such materials. After reviewing the PN information, FDA may determine that the regulated articles should not be allowed into the United States or may allow conditional import of articles subject to testing and release at the port of entry. In addition, during FDA review at the port the regulated articles must meet all requirements of the FD&C Act and 21 CFR before they are released by FDA to the importer.

Importers of record (individuals or companies) for excipients can contract with a broker to transmit PN information and other documents for them. In this case, the submitter is the person responsible for providing the information, but the broker is the transmitter. Brokers are licensed private individuals or companies regulated by CBP who aid importers and exporters in moving merchandise through CBP. Brokers provide the proper paperwork and payments to CBP for clients and charge a fee for this service. Before brokers apply for a license, they must pass the customs broker examination.

FDA currently uses its Operational and Administrative System for Import Support (OASIS)[11] for making its admissibility determinations to ensure the safety, efficacy, and quality of the foreign-origin products for which FDA has regulatory responsibility under the FD&C Act. OASIS is integrated with CBP's ACS and FDA's PNSI systems to receive information related to imported articles.

US Customs and FDA storage areas at the Port of Arrival may not strictly be in compliance with storage conditions required for certain excipients. Importers of record and brokers who represent importers must ensure that the products are released from Customs and FDA inspection as soon as possible. If release is delayed, FDA generally allows removal by Customs and FDA and quarantine in the importer's warehouse until release. FDA staff who review imports at the port of entry are trained to understand that pharmaceutical excipients must be stored under defined conditions. The manufacturer, the importer on record in the importing country, and brokers have a responsibility in working with Customs and FDA staff to ensure that the storage conditions do not adversely affect product quality during quarantine and review.

4.8 Traceability

4.8.1 TRACEABILITY

The pedigree of the excipient should be tracked from the manufacturer's storage through the final delivery to customers by means of recorded identification. The entire supply chain should provide full traceability (for example, via lot numbers and shipping documents) in order to allow fast and efficient investigation of any quality issue or product recall. Every entity in the supply chain should also take responsibility from the preceding supplier and pass the product to subsequent intermediaries down to the end user. Therefore, the original excipient manufacturer and subsequent handlers should always be traceable, and the information should be available both downstream and upstream in the supply chain. All parties to the excipient supply chain should ensure that the excipient is strictly handled according to GDP at every stage.

To ensure the integrity of the supply chain, intermediaries should use contracts, agreements, inspections, and audits downstream and upstream to monitor compliance with GDP principles. When multiple entities constitute the supply chain for each single batch of excipient, each entity should provide its own supplier's certification documentation (see *Appendix: Definitions and Acronyms*) that represents their manufacture or receipt of the excipient batch through release to the subsequent entity. The total of each entity's supplier certification documentation should represent the entire supply chain from original excipient manufacture through use in the final drug product.

4.8.2 TRACEABILITY-RELATED DOCUMENTS

To ensure traceability, all entities in the supply chain should have clear definitions about the shipping documents to be expected with every delivery. At a minimum the documents for every delivery should provide the following information:

- Name and grade of the excipient
- Lot number(s) assigned by the original excipient manufacturer (see *Section 3.4.2 Repackaging and Labeling Batches*)
- Quality and compliance data (e.g., COA) of the excipient
- Origin of the excipient (manufacturer and manufacturing site)
- Original excipient COA(s) (see *Section 3.4.5 Certificates of Analysis*)
- Entity and site of repackaging (when performed), including opening or relabeling the original excipient manufacturer's packaging for any purpose
- Date of shipment and carrier
- Consignor and consignee

A copy of the COA also should accompany the shipment (see *Significant Change Guide for Bulk Pharmaceutical Excipients* 〈1195〉 and *Bulk Pharmaceutical Excipients—Certificate of Analysis* 〈1080〉).

In the event of repackaging from the original excipient manufacturer's package into another container (including any breach or labeling that does not result in a new package), the identity and address of the repackaging entity should be included in the shipping documents.

Additional data resulting from analyses conducted by entities other than the laboratory of the original excipient manufacturer should be provided, along with a clear indication of the source. Quality documents should facilitate traceability back to the

[11] FDA. Operational and Administrative System for Import Support (OASIS). 2009.

original manufacturer with contact information. The COA issued by the manufacturer should indicate which results were obtained by testing the original material and which results were obtained by other means. A distributor should not change the original title and data of the COA or other quality documents. Whenever possible, the original excipient manufacturer's documentation should be used, or data transcription should be verified. The original manufacturing site should be identified on the COA.

If any lot mixing is carried out, COAs from manufacturers are no longer valid, and the distributor should perform analyses in its own laboratory or at an approved and qualified contract laboratory. The distributor should supply a COC, and if the blended lot has not been retested, the distributor should inform the customer that the material is a mixture of different original excipient manufacturers' lots, provided that all other repackaging and storage activities are carried out according to GDP.

SECTION 5: EXCIPIENTS USED IN PHARMACY COMPOUNDING

Although analytical testing of incoming components by the compounding pharmacy to confirm quality attributes stated in the COAs is ideal, generally because of resource limitations, compounding pharmacists rely upon the distributors of excipients for assurance of quality and pedigree. Additional guidance on the quality attributes of excipients received by compounding pharmacies can be found in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩. In certain instances *USP–NF* handles compounded preparations differently than commercially manufactured lots. For example, expiration dates are assigned to commercially manufactured products, and beyond-use dates are assigned to compounded preparations (see *General Notices 10.40.100.1 Compounded Preparations*). A similar situation is needed for bulk pharmaceutical excipients as not all excipients that are useful in compounding are listed in official compendia (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

State boards of pharmacy regulate pharmacy compounding. USP standards are provided in *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩, *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, and *Quality Assurance in Pharmaceutical Compounding* ⟨1163⟩.

APPENDIX: DEFINITIONS AND ACRONYMS

Acceptance Criteria: The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.
ACS: Automated Commercial System.
Adulterated Material: A material that fails to conform to its purported quality standard or is intentionally contaminated, diluted, or substituted for another substance or which was not manufactured, processed, packaged, distributed and held in conformance with current good manufacturing practice.
Audit: An assessment of a system or process to determine its compliance with the requirements of a particular standard of operation. See also External Audit, Internal Audit, and Third-Party Audit.
Batch (Lot): A defined quantity of processed excipient which can be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production based on time or quantity (e.g., vessel's volume, one day's production, etc.).
Batch Number (Lot Number): A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.
Batch Process: A manufacturing process which produces the excipient from a discrete supply of raw materials processed through discrete unit operations in one mass.
Batch Record: Documentation that provides a history of the manufacture of a batch of excipient.
Blending (Mixing): Intermingling different conforming grades into a homogeneous lot.
Broker: An entity that acts as an intermediary between a buyer and a seller of products or services. Brokers neither buy nor take possession of the products or services.
Calibration: The demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.
CBP: Customs and Border Protection.
CEP (Certificate of Suitability to the *European Pharmacopoeia*): Certification granted to individual manufacturers by the European Directorate for the Quality of Medicines when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.
CFR: Code of Federal Regulations.
CFR (Cost and Freight, Named Destination): (Incoterm) Seller must pay the costs and freight to bring the goods to the port of destination. However, risk is transferred to the buyer once the goods have crossed the ship's rail (maritime transport only).
cGMP: Current good manufacturing practices.
CIF (Cost, Insurance, and Freight, Named Destination): (Incoterm) Same as CFR except that the seller must, in addition, procure and pay for insurance for the buyer.

CIP (Carriage and Insurance Paid, Named Destination): (Incoterm) The containerized transport or multimodal equivalent of CIF. Seller pays for carriage and insurance to the named destination, but risk passes when the goods are handed over to the first carrier.

Closed-Container Distributor (Pass-Through Distributor): A distributor who sells only products that are tested, packaged, and sealed in the containers provided by the original manufacturer.

Closed System: A system that is isolated from its surroundings by a boundary so that no material can be transferred across it.

COA (Certificate of Analysis): A document that reports the results of a test of a representative sample drawn from the batch of material that will be delivered.

COC (Certificate of Conformance): A document that certifies that the supplied goods or service meets the required specifications. Also known as Certificate of Conformity and Certificate of Compliance.

Commissioning: The introduction of equipment for use in a controlled manner.

Compounding: The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice (defined in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Consignee/Consignor: Person or firm (usually the seller) who delivers a consignment to a carrier for transportation to a consignee (usually the buyer) named in the transportation documents.

Contamination: The undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

Continuous Process: A manufacturing process that continually produces the excipient from a continuous supply of raw material.

Contract Giver: A person or organization letting a contract.

Contract Acceptor: A person or organization accepting the terms of a contract and thereby agreeing to carry out the work or provide the services as specified in the contract.

Critical: A process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

Critical to Quality: See *Quality, Critical.*

Cross-Contamination: Contamination of a material or product with another material or product.

Customer: The organization that receives the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

Deviation: Departure from an approved instruction or established standard.

Distributor: An entity that buys products from a manufacturer, takes possession of those products, and resells them to another party or parties. An essential characteristic of a distributor is the order of these transactions. Distributors buy products (i.e., hold inventory) before making sales.

Drug Master File (DMF): Detailed information about the manufacture of an excipient that is submitted to the US FDA.

Drug (Medicinal) Product: The dosage form in the final immediate packaging intended for marketing.

Drug Substance: Any substance or mixture of substances that is intended for use in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

Economically Motivated Adulteration: The fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production for economic gain.

Electronic Signature: A computer data compilation of any symbol or series of symbols, executed, adopted, or authorized by an individual and intended to be the legally binding equivalent of the individual's handwritten signature.

Excipient: Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

Excipient Pedigree: Includes documentation of suitable excipient good manufacturing practices applied by the excipient manufacturer and suitable good distribution practices. See IPEC *Excipient Pedigree White Paper.*

External Audit: (See also *Audit, Internal,* and *Third-Party Audit.*) An audit carried out typically on behalf of an excipient manufacturer's customer by a person or organization that is not the manufacturer or the customer.

Expiry (Expiration) Date: The date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

FCA (Free Carrier, Named Place): The seller hands over the goods, cleared for export, into the custody of the first carrier (named by the buyer) at the named place. This term is suitable for all modes of transport, including carriage by air, rail, road, and containerized/multimodal transport (also called *roll on–roll off*).

FDA: Food and Drug Administration.

FD&C Act: Food, Drug, and Cosmetic Act.

FOB (Free on Board, Named Loading Port): The classic maritime trade term according to which the seller must load the goods on board the ship nominated by the buyer, and cost and risk are divided at ship's rail. The seller must clear the goods

for export. The purchaser is then responsible for all further costs associated with transport, importation, and storage until the shipment reaches its destination. The term also is applied to air transport when the seller is not able to export the goods according to the time schedule detailed in the letter of credit. In this case the seller allows a deduction equivalent to the carriage by ship from the air carriage. FOB also can be qualified in other ways. For example, *FOB Factory Gate* means that title and responsibility change as soon as the shipment leaves the supplier's premises.
Forwarding Agents (Freight Forwarders): Agents who assist other organizations or individuals in moving cargo to a destination and are familiar with the import and export rules and regulations of their own and foreign countries, the methods of shipping, and the documents related to foreign trade.
Freight Forwarder: See *Forwarding Agent.*
GDP: Good distribution practices.
GMP: Good manufacturing practices.
Headspace: The volume left at the top of an almost-filled container before sealing.
HACCP (Hazard Analysis Critical Control Point): Hazard Analysis and Critical Control Points has seven principles established by the National Advisory Committee for Microbiological Criteria for Foods to control product safety.
Importer: Either the US owner or consignee at the time of entry into the United States or the US agent or representative of the foreign owner or consignee at the time of entry into the United States who is responsible for ensuring that goods offered for entry into the United States are in compliance with all laws affecting the importation.
Impurity: A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.
Independent: In the context of internal audits, the quality of being free from any influence, economic or otherwise, from the group, department, or organization under audit.
In-Process Control: Checks performed during production in order to monitor and if necessary to adjust the process to ensure that the material conforms to its specifications. The control of the environment or equipment also can be regarded as a part of in-process control.
In-Process Control/Testing: Checks performed during production to monitor and, if appropriate, to adjust the process to ensure that the intermediate or excipient conforms to its specification.
Intermediate: Material that must undergo further manufacturing steps before it becomes an excipient.
Internal Audit: An audit conducted by an employee of the organization or by an individual from outside the organization, but on behalf of the organization, to determine the effectiveness of a system. (See: *Audit*, *External Audit*, and *Third-Party Audit*).
International Nonproprietary Name: International Nonproprietary Names (INN) facilitate the identification of pharmaceutical substances or active pharmaceutical ingredients. Each INN is a unique name that is globally recognized and is public property. A nonproprietary name also is known as a generic name.
ISO: International Organization for Standardization.
Lot: See *Batch.*
Labeling: The affixing to a container or vessel of a tag or document that contains information about that container and its contents.
Manufacturer/Manufacturing Process: All operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.
Master Production Instruction (Master Production and Control Record): Documentation that describes the manufacture of the excipient from raw material to completion.
Material: A general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.
Nonconforming Material: A material that is deficient in a characteristic, product specification, process parameter, record, or procedure that renders its quality unacceptable, indeterminate, or not according to specified requirements.
OASIS: Operational and Administrative System for Import Support.
Original Excipient Manufacturer: Organization responsible for manufacturing, under appropriate GMPs, the excipient(s) distributed and addressed by this chapter.
Packaging/Repackaging Distributor: A distributor who transfers products from the original packaging or transport vessel(s) provided by the original manufacturer into alternative packaging and sells the products in the alternative packages. See *Distributor* and *Repackager.*
Primary Container–Closure System: The packaging components that come into direct contact with the excipient in the closed, sealed package during storage and transport.
Packaging Material: A material intended to protect an intermediate or excipient during storage and transport.
Packaging: The container and its components that hold the excipient for storage and transport to the customer.
Pass-Through Distributor: See *Closed-Container Distributor.*
PN: Prior notice.
PNSI: Prior Notice Systems Interface.
Primary, Secondary Packaging: See *Packaging/Repackaging Distributor* and *Primary Container—Closure System*. Packaging materials which do not come into contact with the excipient during the normal course of storage and transport of the excipient.

Production: Operations involved in the preparation of an excipient from receipt of raw materials through processing and packaging of the excipient.
QbD (Quality by Design): A systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management. It means designing and developing products and manufacturing processes to ensure a predefined quality.
QMS: Quality management system.
Quality Assurance (QA): The total of the organized arrangements made to ensure that all excipients are of the quality required for their intended use and that quality systems are maintained. See *Quality Unit*.
Quality Control (QC): Checking or testing that specifications are met. See *Quality Unit*.
Quality, Critical: Describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.
Quality Management System (QMS): Management system that directs and controls a pharmaceutical company with regard to quality.
Quality Manual: Describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes. Document specifying the quality management system of an organization.
Quality Unit: See also: *Quality Control* and *Quality Assurance*. A group within a larger organization that is responsible for monitoring and ensuring all aspects of quality. Current industry practice generally divides the responsibilities of the quality control unit (QCU), as defined in the cGMP regulations, between quality control (QC) and quality assurance (QA) functions. QC usually involves (1) assessing the suitability of incoming components, containers, closures, labeling, in-process materials, and the finished products; (2) evaluating the performance of the manufacturing process to ensure adherence to proper specifications and limits; and (3) determining the acceptability of each batch for release. QA primarily involves (1) review and approval of all procedures related to production and maintenance, (2) review of associated records, and (3) auditing and performing/evaluating trend analyses.
Quarantine: The status of materials isolated physically or by other effective means pending a decision about their subsequent approval or rejection.
Raw Material: A general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.
Recall: See *Retrieval*.
Record: A document stating results achieved or providing evidence of activities performed. The medium can be paper, magnetic, electronic or optical, photographic, another medium, or a combination thereof.
Reevaluation Date (Retest Date, Re-evaluation Interval): The date when the material should be reexamined to ensure that it is still in conformity with the specification.
Recommended Re-evaluation Date: The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. It differs from the Expiration Date because the excipient can be re-evaluated to extend the length of time the material can be used, if supported by the results of the evaluation and appropriate stability data.
Repackager: A person or organization that takes an excipient from the original manufacturer's container and repackages it into different containers. See also *Distributor* and *Packaging/Repackaging Distributor*.
Repackaging: Removal of the excipient from its original container (combination of secondary and/or primary packaging), and transfer to another container.
Reprocessing: Introduction of previously processed material that did not conform to standards or specifications back into the process and repetition of one or more necessary steps that are part of the normal manufacturing process.
Retrieval (Recall): Process for the removal of an excipient from the distribution chain.
Reworking: Subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.
Secondary, Primary Packaging: See *Primary, Secondary Packaging*.
Senior Management: See *Top Management*.
Significant Change: A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.
Specification: The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.
Stability: Continued conformity of the excipient to its specifications.
Stable Process: A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP and customer specifications) and thus is acceptable for its intended use.
Subcontractor: A person or organization that undertakes work or services on behalf of a different person or organization that in turn is contracted to undertake work or provide services from the original contract giver.
Supplier's Certification Documentation: Specific information and data associated with a single batch of an excipient. Its accuracy is certified by the business entity that has had control of the same single batch of excipient. Supplier's Certification Documentation includes both quality and supply chain data and information. The methods and processes that derive the included data and information should be understood and controlled, and all data and information sources should be traceable. All enti-

ties that take possession and responsibility for the excipient batch should provide Supplier's Certification Documentation including the original excipient manufacturer, all distributors, and all repackagers. Special attention and clarity should be applied within the Supplier's Certification Documentation in any event that breaches the original manufacturer's packaging and/or labels (including addition of new labels).

Third-Party Audit: An audit conducted by an individual from outside the organization and who is neither a supplier nor customer of the organization, e.g., a certification body, to determine the effectiveness of a system.

Top Management: Person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and depends on how the quality management system is organized.

Traceability: Ability to determine the history, application, or location that is under consideration, e.g., origin of materials and parts, processing history, or distribution of the product after delivery.

Trader: An entity that buys products from a manufacturer, may or may not take possession of the products, and resells them to another party or parties.

NOTE: In the case of traders, the sale usually is made before product purchase.

User: A person or organization that uses pharmaceutical excipients to manufacture pharmaceutical intermediates or finished products.

Validation: A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

〈1207〉 PACKAGE INTEGRITY EVALUATION—STERILE PRODUCTS

1. INTRODUCTION

This chapter provides guidance on the integrity assurance of nonporous packages intended for sterile pharmaceutical products. Background instruction is provided on the topics of leaks, leakage rate, and package sealing/closure mechanisms. Explanation is given as to how packages that conform to specified leakage limits help to ensure the contained product meets and maintains sterility and relevant physicochemical specifications. The integration of package integrity assurance as a key component of the entire product life cycle is stressed. Guidance in the selection, validation, and use of leak test methodologies as well as package seal quality tests is included. Detailed recommendations are presented in three subchapters listed below:

- *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* 〈1207.1〉
- *Package Integrity Leak Test Technologies* 〈1207.2〉
- *Package Seal Quality Test Technologies* 〈1207.3〉

At the end of this chapter, the *Glossary* section defines terms as they are used in the context of this set of four general information chapters.

The term product–package refers to the container–closure system plus the product contents. The container–closure system consists of the primary packaging components, those components that are or may be in intimate contact with the product, as well as secondary packaging components vital to ensuring correct package assembly, for example, the aluminum cap used to seal a stoppered vial package.

Suitable container–closure systems adequately store and protect the contained pharmaceutical product. Thus, sterile product–package integrity is the ability of a sterile product container–closure system to keep product contents in, while keeping detrimental environmental contaminants out. Specifically, leaks of concern for sterile product–packages include the following three categories described in *Table 1*. In other words, the leaks of concern for a given product–package are a function of the degree of package protection demanded by the product to ensure that all relevant product physicochemical and microbiological quality attributes are met through product expiry and use.

Table 1. Product Quality Risks Posed by Leaks of Concern

Leaks of Concern	Product Quality Risks Posed by Leaks
Capable of allowing entry of microorganisms	Failure of product sterility quality attribute
Capable of allowing escape of the product dosage form or allowing entry of external liquid or solid matter	Failure of relevant product physicochemical quality attributes
Capable of allowing change in gas headspace content. For example, loss of headspace inert gases (e.g., nitrogen), loss of headspace vacuum, and/or entry of gases (e.g., oxygen, water vapor, air).	Failure of relevant product physicochemical quality attributes and/or hindrance of product access by the end-user

Package integrity is synonymous with container–closure integrity; these terms are used interchangeably throughout this chapter. In the past, to say a sterile product–package has container–closure integrity commonly meant that the package either had passed or was capable of passing a microbiological challenge test. This guidance chapter defines the concept of container–closure integrity more broadly, encompassing the absence of all package leaks that risk product quality. By this definition, a package is considered to have integrity if it allows no leakage greater than the product–package maximum allowable leakage limit. In other words, the largest and smallest leaks of concern are absent.

Leakage differs from permeation. Leakage is the unintentional entry or escape of matter (solids, liquids, or gases) through a breach in a package wall or through a gap between package components. Leakage can also refer to the leaking matter itself. Leakage flow rate is a function of the absolute and/or partial pressure gradient of leaking matter that exists across the package barrier. In the context of this chapter, permeation is the passage of fluid (e.g., gas) into, through, and out of a nonporous package wall. Permeation, not leakage, occurs when only a small fraction of molecules is able to move through a barrier by way of any one hole. A nonporous package is able to permit permeation, but not the volumetric flow of air. Package permeation test methodologies fall outside of the chapter scope and are not described.

Package integrity tests are leak test methods. A leak test is a method that detects the presence of (and in some cases, the magnitude or location of) a package breach or gap.

Package seal quality tests are techniques used to characterize and monitor the quality and consistency of a package seal or closure system parameter, which can influence the package's ability to maintain integrity. An example is the peel force test widely used to test seal strength. Seal quality tests are not leak tests but can play a valuable role by monitoring a characteristic of the seal itself, the package materials, the package components, and/or the processes required to create the seal or closure mechanism.

Integrity test methods vary not only in their application and detection limit, but also in terms of detection range, precision, and specificity, for example. No one test is appropriate for all packages or for all leak testing applications. Selection criteria for package integrity test methods, as well as method comparison aids, are presented to guide the user in the selection process.

Leak tests, even many commonly recognized industry standard testing approaches, require optimization and validation for each product–package application. A science- and risk-based approach may allow some tests to be leveraged for broader application under certain circumstances. For example, small differences in product formulation or package design and materials may permit the use of one test for multiple product–packages. Package integrity verification plays an important role throughout the product life cycle, starting with product development and continuing through marketed product stability studies.

2. SCOPE

The package integrity leak tests and seal quality tests outlined in this chapter apply to the testing of both large and small volume container–closure systems for sterile pharmaceutical dosage forms. Such package systems include, but are not limited to, the following examples.

- Vials or bottles closed with elastomeric closures or screw-thread caps
- Form-fill-seal plastic or glass ampules
- Syringes or cartridges
- Flexible bags or pouches
- Packages for some drug/device combination products (e.g., packages cased inside autoinjectors)

Outside the chapter scope are packaging systems and processing equipment used in the preparation, storage, and manufacture of sterile pharmaceutical products. Examples include containers for active pharmaceutical ingredients and containers for product intermediate or final bulk product.

Outside the chapter scope are packages used for sterile diagnostic products or medical devices, and some packages used for sterile drug/device combination products.

Outside the chapter scope are products having a primary package composed of porous barrier package materials, i.e., materials designed with pores or openings to allow volumetric air flow while preventing airborne microbial contamination.

3. LEAKS AND LEAKAGE RATE

Leaks are commonly conceptualized as holes of a defined diameter, or channels of distinctive diameter and length, although leaks that occur naturally are generally complex, multicavity tortuous paths and are rarely uniform in size or shape. Even artificially created leaks such as laser-drilled defects (also called holes) used for leak-test method development and validation are irregular in size, shape, and depth. When stating the size of a leak, it is important to define the measurement approach. In some cases leaks are measured dimensionally, but quite often, leak size is determined based on gaseous leakage rate. For example, a package wall laser-drilled defect having a nominal diameter of 5 ± 2 µm may have been size-certified by matching the airflow rate through the drilled defect to that of a 5 ± 2 µm hole present in a thin metal plate reference standard when pressurized with dry air at specified differential pressure and temperature conditions.

Gaseous leakage rate is a measure of the rate of gas flow (in mass or volume units) that passes through a leak path under specific conditions of temperature and the concentration or pressure differential across the barrier wall. Therefore, gaseous leakage rate has dimensions of pressure multiplied by volume, divided by time. The international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$). These leakage measurement units refer to the quantity of leaking gas ($Pa \cdot m^3$) per unit of time. When a leakage rate is described and no test conditions are noted, standard conditions of one standard atmosphere differential pressure with dry air at 25° are assumed. For a more complete discussion of gaseous leakage rates and units of measure refer to reference *1*.

Unintentional leaks in packages are often detected or sized using gas as a tracer element. Given a situation in which a tracer gas partial pressure difference exists across the package barrier wall and no absolute pressure difference exists, gaseous leakage is predominantly diffusional in nature as the tracer gas moves from a region of higher concentration to a region of lower con-

centration. For example, the headspace in a vial package low in oxygen concentration and at ambient pressure at time of closure will exhibit a rise in headspace oxygen concentration over time as a function of diffusion rate (relative to package leak size) plus permeation rate (relative to permeability through the package). Such a change can be monitored instrumentally, as is discussed later in the chapter.

Given the situation in which an absolute pressure difference exists across the package barrier wall, gas leakage through package gaps is more rapid (flux being primarily convective) as gas moves from the higher pressure region to one of lower pressure. For most package materials, the permeation rate of the gas of concern through the package wall is insignificant in comparison. In this example, the rate of leakage into the package is determined by monitoring the change in headspace absolute pressure as a function of time.

For many instrumental leak test methods described in this chapter, gas flow into or out of package leaks is induced by exerting a pressure gradient across the package. In this way, a wide range of leak types and sizes can be identified.

Liquid leakage rate is a measure of the volume of liquid that moves through a leak path as a function of time under specified conditions of temperature and absolute differential pressure across the barrier wall. Liquid movement through a leak path occurs only when leak size/shape, package materials of construction, the absence of leak obstruction, tracer liquid composition, and test parameters all work together. All sterile product–packages within the chapter scope are intended to prevent liquid leakage and block microbial entry, thus it is the absence of liquid leakage flow or the absence of liquid in the leak path, rather than the rate at which liquid leakage occurs that is typically verified in relevant package leak tests. Liquid leakage rate measurements are only useful in measuring larger size leaks.

4. CLOSURE TYPE AND MECHANICS

An understanding of closure mechanics makes it possible to better characterize, monitor, and test packages for integrity. This section discusses various closure systems and how each type functions to ensure package integrity.

4.1 Physically Mated Closures

Closure can be achieved by the close physical mating of two surfaces that often are dissimilar in material composition. Examples include the interference fit of a plunger inserted in a syringe barrel, the compression fit of an elastomeric closure capped onto a vial finish surface, and the application force of a screw-cap torqued onto a bottle. Physically mated surfaces are not bonded together; therefore, a tiny gap exists even between well-closed components. Nevertheless, when properly designed and assembled, closure systems fit together such that liquid leakage (and microbial ingress) is prevented and gas migration is limited. Regarding screw-thread closures such as those used for ophthalmic-product dropper bottles, the barrier to leakage is afforded by inner cap surfaces pressing against the package opening, in some cases aided by a secondary gasket or plug. The winding path afforded by the threads of a screw-cap does not provide an optimal barrier to gas or liquid leakage, or to microbial ingress in the event of liquid presence in the cap threads.

4.2 Physicochemically Bonded Closures

Seals are formed when two similar or dissimilar surfaces are physicochemically bonded together. One example is the formation and sealing of contiguous containers from a single material (e.g., glass or plastic ampules). In other form, fill, and seal systems, sheets of material are mated by means of a heat or ultrasonic welding process. Two dissimilar materials may be joined using an intermediate bonding material. Fully fused seals inherently block liquid leakage and microbial ingress; however, gaseous leakage and permeation may occur.

4.3 Multiple-Dose Package Closures

Multiple-dose package closures are designed to allow product access while limiting microbial ingress and product leakage between doses. For example, elastomeric closures for parenteral products requiring needle-puncture access are designed to offer reseal protection against microbial ingress and product leakage. Some multiple-dose ophthalmic dosage form package closures are designed with filters, plugs, or other mechanical means that allow the product to be dispensed while restricting microbial ingress and product leakage. For example, ophthalmic product–package closure systems have been designed to automatically pinch shut between intermittent dosing to limit microbial entry and product loss.

5. PRODUCT–PACKAGE QUALITY REQUIREMENTS AND THE MAXIMUM ALLOWABLE LEAKAGE LIMIT

As noted in *Introduction*, package integrity is necessary to maintain product critical quality attributes within physicochemical label-claim specifications and to ensure product sterility until time of use. Detrimental contaminants include microorganisms and any substances that threaten patient safety or product quality. Product leakage can cause a product to fail content or potency specification limits. For certain products to maintain product physicochemical stability, the package needs to maintain a

headspace of nonreactive gases and/or low water vapor content, sealed under atmospheric or reduced-pressure conditions. Headspace vacuum conditions may also be necessary to facilitate product ease of use, e.g., product reconstitution using a diluent injected into the container.

Most package types display very low but definite gaseous leakage flow through the gap that exists even between well-fitted closures. Therefore, it is not practical to require that packages be absolutely leak-free. Rather, it is the significance of leakage in relation to product quality that needs to be considered. In other words, the package should not permit leakage beyond the product's maximum allowable leakage limit. Such leaks of concern should be absent.

Identifying the maximum allowable leakage limit for a product–package is a science- and risk-based decision. The smaller the leak path, the less likely the product can escape and the less likely microorganisms or other contaminants can enter. Eventually, leak paths may be so small that only headspace gas exchange is realistically possible. Package construction and assembly, package contents, and the range of environments a given product–package may be exposed to during its life cycle are to be considered when specifying the maximum allowable leakage limit.

There are two major product–package quality requirement categories used when specifying the maximum allowable leakage limit and one subcategory that applies only to multiple-dose packages. These are discussed below.

5.1 Sterility and Product Formulation Content must be Preserved; Gas Headspace Content Preservation is not Required

This category includes product–packages for which the maximum allowable leakage limit correlates to the prevention of product formulation escape, or product contamination by external liquid or solid matter or by microorganisms, while gas headspace preservation (i.e., ambient pressure air) is of no concern. The smallest leak paths that allow only limited gas exchange are irrelevant as they pose no real risk to product quality.

Considerable published research exists exploring rigid package leaks and their relationship to risk of aqueous solution leakage and/or microbial ingress. A key study series found orifice leaks of approximately 0.1 μm in nominal diameter (using micropipettes) placed in rigid packages demonstrate a small risk of aqueous liquid passage, while orifice leaks as small as approximately 0.3 μm in nominal diameter first demonstrate some risk of microbial ingress by liquid challenge (*2,3*). For a summary of similar studies comparing risks of microbial ingress and liquid leakage to leak size and type, the reader may refer to reference *4*.

Given this body of evidence, a maximum allowable leakage limit of less than 6×10^{-6} mbar · L/s (measured by helium mass spectrometry in the vacuum mode) can be adopted for products in this category packaged in rigid container–closure systems. This leakage rate equates to the presence of an orifice of nominal diameter of between 0.1 and 0.3 μm. At this leakage rate, the probability of microbial ingress was determined to be <0.10 (*2*). Selecting this conservative maximum allowable leakage limit will ensure a low risk of microbial ingress and liquid leakage and can eliminate the need to perform additional microbial ingress or liquid challenge studies as a function of leak size.

For other container systems such as those made using flexible materials or those with complex, lengthy closure/seal interfaces, or those meant to contain a product of markedly greater leakage potential, the risk of microbial ingress or liquid passage through leak paths is not as widely publicized or perhaps understood. In such cases, where the relationship between defect size/type to the risks of microbial ingress and/or liquid passage is less prescriptive, a study exploring these relationships could be useful. Test results can be used to establish a meaningful maximum allowable leakage limit for the given product–package system, which can be employed for package integrity verification by other validated leak test methods of choice. Once established, this maximum allowable leakage limit can be applied to similar product–packages with appropriate justification.

Finally, before classifying a product–package in this category, one may consider the potential impact of product life cycle processing, storage, distribution and use scenarios on package integrity. For example, elastomeric closures have been found to shrink and lose their viscoelastic properties during ultra-cold storage (<80°) to such an extent that gas influx into stoppered vial packages may occur. During warming, package closure is restored, trapping gases and notably raising internal package pressure. In such cases, gas headspace preservation may be a product quality concern, even if the product does not require specific headspace content or pressure for optimum stability.

5.2 Sterility, Product Formulation Content, and Gas Headspace Content must be Preserved

The second category includes product–packages for which the package should prevent product formulation escape and product contamination by external liquid or solid matter or by microorganisms, but in addition, the package must preserve the gas headspace content or absolute pressure. Preservation of headspace content and/or pressure is needed to maintain product stability within physicochemical specification limits, and/or to aid end-user product access. Thus, the maximum allowable leakage limit for such products is likely more stringent than that described in the first category. Although outside the chapter scope, the influence of gas permeation through the package itself may also need to be considered when establishing the maximum allowable leakage limit as permeation plus leakage can impact package headspace content.

The maximum allowable leakage limit for products in this category may be expressed in terms of the maximum allowable package headspace content or pressure change as a function of time. This limit, established for each product–package system, may be applied to other similar product–packages systems with appropriate justification.

5.3 Sterility must be Preserved; Product Access is Required

This subcategory represents a quality requirement applicable only to multiple-dose product–packages included in either of the above two categories. Once the product–package has reached the end-user and the closure has been activated or otherwise compromised to allow dosage access and delivery, the maximum allowable leakage limit at this life cycle phase (called the in-use maximum allowable leakage limit) is defined in terms of microbial ingress and product loss prevention between and during dosage access. For example, elastomeric closures of multiple-dose vials or cartridges containing antimicrobial-preserved parenteral products are designed to afford reseal protection against microbial ingress and product formulation leakage as product is accessed via needle puncture as well as between doses.

To establish the in-use maximum allowable leakage limit for multiple-dose package closure systems, a study will likely be required to explore the relationship between product access attempts, product loss risk and/or microbial ingress risk, versus leakage measurement by an alternative adequately sensitive leak test method of choice. These data can be used to establish a meaningful in-use maximum allowable leakage limit expressed in units of measure reflective of the preferred leak test method and that will assure that product loss and microbial ingress risk during use is minimal. The in-use maximum allowable leakage limit established for a given product–package may be applied to other similar product–packages systems with appropriate justification.

6. INHERENT PACKAGE INTEGRITY AND THE PACKAGE INTEGRITY PROFILE

Inherent package integrity is the leakage characteristic of a well-assembled container–closure system using no-defect package components. Inherent package integrity is first determined during product–package development and qualification and is a measure of the leak tightness of a container–closure system, given anticipated variables of material composition, dimension, processing, and assembly. Inherent package integrity may also be determined as a function of anticipated final product storage, distribution, and use. Acceptable inherent package integrity for a container–closure system conforms to the specific product–package maximum allowable leakage limit. Inherent package integrity is expressed in terms that allow a meaningful comparison to the maximum allowable leakage limit.

Confirmation that the inherent package integrity conforms to the maximum allowable leakage limit is the first step in product–package integrity verification. Verification of package integrity continues throughout the product life cycle. The package integrity profile is an ongoing database of product life cycle package leak and seal quality test results. This profile provides information regarding package integrity given operative variations in package component design and material; package assembly and processing; and product storage, distribution, and stability. These concepts are more fully explored in *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* 〈1207.1〉, *Test Instrument Qualification, Method Development, and Method Validation.*

In summary, a product–package system having integrity is one in which the inherent package integrity conforms to the required product–package maximum allowable leakage limit. Further, the package integrity profile database operates as a risk management tool to ensure that finished product container–closure systems are intact, able to block microbial ingress, restrict loss of product contents including critical headspace gases, and prevent entry of detrimental gases or other substances, thus ensuring that the product meets all relevant physicochemical and microbiological label-claim specifications through expiry and final end-use.

REFERENCES

1. Jackson CN, Sherlock CN, Moore PO, editors. In: Nondestructive testing handbook. 3rd ed. Vol. 1. Leak testing. Columbus, OH: The American Society for Nondestructive Testing; 1997.
2. Kirsch LE, Nguyen L, Moeckly CS, Gerth R. Pharmaceutical container/closure integrity. II: The relationship between microbial ingress and helium leak rates in rubber-stoppered glass vials. *PDA J Pharm Sci Technol.* 1997;51(5):195–202.
3. Kirsch LE. Pharmaceutical container/closure integrity. VI: A report on the utility of the liquid tracer methods for evaluating the microbial barrier properties of pharmaceutical packaging. *PDA J Pharm Sci Technol.* 2000;54(4):305–314.
4. Guazzo DM, Singer DC, Stevens-Riley M, Walker D, Kirsch LE, Buus P, et al. Proposed revisions to general chapter sterile product packaging—integrity evaluation 〈1207〉. Stimuli to the revision process. *Pharmacopeial Forum.* 2014;40(5).

GLOSSARY

For definitions of container, materials of construction, packaging component, packaging system, primary packaging component, and secondary packaging component, see *Packaging and Storage Requirements* 〈659〉. In the context of this chapter and its subchapters, the following definitions relevant to packaging and package integrity methods, seal and closure mechanisms apply. For definitions of specific leak test and seal quality test methods, refer to 〈1207.2〉 and 〈1207.3〉, respectively.

Accuracy: The accuracy of a leak test method is a measure of the ability of the method to correctly differentiate packages that leak above the claimed detection limit from those that leak below this limit (do not leak); a measure of false positive and negative occurrence. Alternatively, for those methods that deliver an outcome that is a direct quantitative measure of

gas leakage rate (or of gas content or pressure), accuracy is a measure of the method's ability to produce an outcome comparable to a true standard, such as a nationally recognized gas standard.

Container–closure integrity: Container–closure integrity is the ability of a package to prevent product loss, to block microorganism ingress, and to limit entry of detrimental gases or other substances, thus ensuring that the product meets all necessary safety and quality standards. Synonymous with *Package integrity*.

Container–closure integrity test: A container–closure integrity test is any package leak test (either physicochemical or microbiological) that detects the presence of a package breach or gap. Some tests may also be able to identify the leak magnitude and/or location. The term container–closure integrity test is synonymous with package leak test or package integrity test.

Container–closure system: See *Packaging and Storage Requirements* ⟨659⟩, *General Definitions, Packaging System*.

Detection limit: The leak test detection limit is a measure of test method sensitivity. The detection limit is the smallest leakage rate (or leak size) that a leak test method can reliably detect. Also called limit of detection.

Deterministic leak test method: A deterministic leak test method is one in which the leakage event being detected or measured is based on phenomena that follow a predictable chain of events. In addition, the measure of leak detection is based on physicochemical technologies that are readily controlled and monitored, yielding objective quantitative data.

In-use maximum allowable leakage limit: See *Maximum allowable leaking limit*.

Inherent package integrity: Inherent package integrity is the leakage rate (or leak size) of a well-assembled container–closure system using no-defect package components. Inherent package integrity is a measure of the leak tightness of a container–closure system, given anticipated variables of material composition, dimension, processing, assembly; package storage, distribution and use.

Leak: A leak is a breach in a package wall or a gap between package components that is capable of permitting the passage of gas, liquid, or solid matter. Leak is synonymous with leak path.

Leakage: Leakage is the unintentional escape or entry of matter (solid, liquid, or gas) through a breach in the package wall or through a gap between package components. Leakage can also refer to the substance that enters or escapes from a compromised package. For example, "Leakage from the cracked container stained the package label."

Leakage rate: Gaseous leakage rate is a measure of the rate of gas flow (in mass or volume units) that passes through a leak path under specific conditions of temperature and absolute pressure or concentration differential. Leakage rate has dimensions of pressure multiplied by volume, divided by time. For example, the international standard SI nomenclature is pascal cubic meter per second ($Pa \cdot m^3 \cdot s^{-1}$). Other common units of measure include standard cubic centimeters per second ($std \cdot cm^3 \cdot s^{-1}$ or sccs) and mbar liters per second ($mbar \cdot L \cdot s^{-1}$). Liquid leakage rate is a measure of the volume of liquid that moves through a leak path as a function of time under specified conditions of temperature and absolute differential pressure across the barrier wall. Liquid leakage rate measurement, possible for larger leak tests, requires that leak size, materials of construction; absence of leak obstruction; tracer liquid composition; and test parameters work together to ensure liquid movement through the leak path.

Linearity: Leak test method linearity is the ability of the method to elicit test results that are mathematically proportional to leak path size or leakage rate.

Master: A master is a package prototype, model, or facsimile made to simulate an actual package in shape and design. Masters may be made of solid plastic or metal, or they may be simply a designated container–closure unit. Masters are designed to simulate a no-leaking package, and are often used in leak test system suitability tests to verify instrument performance.

Maximum allowable leakage limit: The maximum allowable leakage limit is the greatest leakage rate (or leak size) tolerable for a given product–package that poses no risk to product safety and no or inconsequential impact on product quality. The maximum allowable leakage limit for a sterile pharmaceutical dosage form package will ensure the content's sterility, preserve product contents, and prevent entry by detrimental gases or other substances, thus ensuring that the product meets relevant physicochemical and microbiological specifications through expiry and use. For multiple-dose product–packages, the in-use maximum allowable leakage limit is defined as the degree of protection demanded of the closure to limit microbial ingress and product formulation leakage between and during dosage access.

Microbiological challenge test: A microbiological challenge test is a package leak test whereby package integrity is evaluated by exposing containers filled with growth-supportive media to microorganisms suspended in submersion media (a liquid-borne challenge test). Leakage is evidenced by the subsequent growth of the challenge microorganisms in the package contents. Synonymous with microbial challenge test.

Negative control: A negative control is a package with no known leak. Negative controls used for leak test method development and validation studies represent packages optimally assembled using normally processed components. Negative controls should duplicate the container–closure system of the product under integrity investigation. For some methods, it may be necessary for negative controls to simulate test product headspace and formulation content as well.

Nonporous: Nonporous packaging does not have pores or openings to allow volumetric air flow. In other words, NMT a small fraction of molecules is able to pass through any one hole. Nonporous materials may be impermeable, semi-permeable, or permeable to the passage of fluid through the package barrier wall.

Nominal diameter: In the context of this chapter, nominal diameter is a means of expressing the size of a package leak. Package leaks are typically irregular tortuous paths or matrices that cross a package wall or barrier. Such leaks are difficult to size in accurate or meaningful terms. The nominal diameter of a leak path is defined as the width of an orifice of rela-

tively short depth, through which gas (e.g., dry air) at equivalent airflow rate may pass when subject to one atmosphere differential pressure at controlled temperature conditions.

Package integrity: See *Container–closure integrity.*

Package integrity test: See *Container–closure integrity test.*

Package integrity profile: The package integrity profile is a database of product life cycle package leak and seal quality test results that denotes product–package integrity given operative variations in package component design and material; package assembly and processing; and product storage, distribution, and stability.

Package leak test: See *Container–closure integrity test.*

Package seal quality: Package seal quality relates to the consistency of a package seal's performance within required specification limits. Examples of package seal quality attributes include heat seal bond strength and capped vial package residual seal force.

Package seal quality test: A package seal quality test is used to characterize and monitor the quality of a product–package seal or closure system to ensure that package assembly is consistently kept within established limits. Package seal quality tests are not and cannot substitute for leak tests. However, they can provide some assurance of the package's ability to maintain integrity by monitoring a characteristic of the seal itself, the package materials, the package components, and/or the processes required to create the seal or closure mechanism. Examples include the seal peel force test and the capped vial package residual seal force test.

Permeation: Permeation is the passage of fluid into, through, and out of a nonporous package wall. Permeation, not leakage, occurs when the package barrier has no holes large enough for more than a small fraction of molecules to pass through any one hole.

Physicochemical package integrity test: A physicochemical package integrity test is a leak test that detects the presence of a package leak, or detects/measures package leakage rate, via physical or chemical means. All leak test methods that do not use microorganisms for leak detection are physicochemical leak test methods.

Porous: A porous package or package material has pores or openings to allow volumetric air flow while still blocking airborne microbial passage. Examples include coated paper or spun-bonded polyolefin barrier materials that permit package penetration by ethylene oxide gas or steam.

Positive control: A positive control is a package with a known, intentional defect. Positive controls used for leak test method development and validation studies should duplicate study negative controls in terms of materials of construction, package assembly, and component processing. Positive controls are used during leak test method development and validation. Some methods require the use of positive controls during routine testing as well.

Precision: Leak test method precision is a measure of the method's ability to yield reliable, repeatable data. Precision includes repeatability (repeat testing of a single homogeneous test sample population set), ruggedness (within laboratory tests performed by multiple operators on multiple days, using multiple instruments; also known as intermediate precision), and reproducibility (tests among multiple laboratories).

Probabilistic leak test method: A probabilistic leak test method is the converse of a deterministic leak test method, being stochastic in nature. Probabilistic tests rely on a series of sequential and/or simultaneous events, each associated with random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate large sample sizes and rigorous test-condition controls to obtain meaningful results. Typically, sample size and test condition rigor are inversely related to leak size.

Product: The pharmaceutical product includes the pharmaceutical formulation as well as the packaged headspace, which may consist of ambient air or nonreactive gases with specified water-vapor content under full or sub-atmospheric pressure conditions.

Product–package: The product–package includes the primary package with critical secondary components (the container–closure system) plus the packaged product (the contents).

Qualitative measure of analysis: A qualitative measure of analysis for leak testing is a measurement approach based on a subjective evaluation of some quality, attribute, or characteristic of the test sample. Visual inspection is an example of a qualitative measure of analysis.

Quantitation limit: The quantitation limit is a leak test method characteristic defining the lowest leakage rate or leak size that can be differentiated with accuracy and precision under the stated experimental conditions.

Quantitative measure of analysis: A quantitative measure of analysis for leak testing is a measurement approach based on objective, numeric data that either directly or indirectly correlates with leak presence, leak location, or leakage rate. Examples include the mass-of-gas-per-time reading generated by the helium mass spectrometry tracer-gas leak test, or the pressure reading as a function of test time measurement produced by the vacuum-decay method.

Range: The range of a leak test method is that interval between the smallest to largest leak size (or leakage rate) that can be detected by a given leak test method with a suitable level of accuracy and precision.

Robustness: Robustness of a leak test method is the method's ability to accurately identify leaking versus nonleaking packages despite small but deliberate variations in procedural parameters, providing an indication of the method's suitability during normal usage.

Specificity: The specificity of a leak test method is the ability of the method to accurately differentiate leaking and nonleaking packages, despite interfering factors that may cause false detection.

System suitability: System suitability is a manner of ensuring that the leak test method including all factors, which may be subject to variability, that may impact test results (such as instrumentation, analysts, test sample preparation steps, and the test environment) are adequately controlled and maintained in such a fashion that the method is rugged and robust.
System suitability test: A system suitability test is a test to verify that the leak test method and all key factors that may impact test results are correctly controlled and set prior to method performance.
Tortuous path: As applied to leaks, a tortuous path is a convoluted, complex leakage pathway. Most naturally occurring leaks, such as cracks and tears, are tortuous in nature, rather than pristine holes. As applied to sealing mechanisms, a sealing material that has tortuous barrier qualities can block microbial entry. [NOTE—The winding path afforded by the threads of a screw-cap (e.g., an ophthalmic dropper bottle closure) does not provide an optimal barrier to gas or liquid leakage, nor does it provide an optimal barrier to microbial ingress in the event of a liquid presence in the cap threads.]
Type defect: A type defect is a positive-control package that represents realistic package flaws. Type-defect positive controls may be included in leak test method feasibility and development studies before method validation. An example of a type defect is a heat seal wrinkle or a loose cap. Type defects are inherently irregular in size and shape and are often described qualitatively instead of being described in terms of leak size or leakage rate.

⟨1207.1⟩ PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE—TEST METHOD SELECTION AND VALIDATION

1. INTRODUCTION
2. PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE
   - 2.1 Development and Validation
   - 2.2 Product Manufacturing
   - 2.3 Commercial Product Stability
3. TEST METHOD SELECTION CRITERIA
   - 3.1 Package Contents
   - 3.2 Package Design, Materials of Construction, and Mechanics
   - 3.3 Closure Type and Mechanics
   - 3.4 Maximum Allowable Leakage Limit
   - 3.5 Deterministic or Probabilistic Methods
   - 3.6 Physicochemical or Microbiological Methods
   - 3.7 Method Outcome
   - 3.8 Quantitative or Qualitative Methods of Analysis
   - 3.9 Leak Test Detection Limit
   - 3.10 Leak Test Method Range
   - 3.11 Nondestructive or Destructive Methods
   - 3.12 Off-Line or On-Line Methods
4. TEST INSTRUMENT QUALIFICATION, METHOD DEVELOPMENT, AND METHOD VALIDATION
   - 4.1 Instrumentation and Equipment Qualification
   - 4.2 Method Development and Validation
   - 4.3 System Suitability
   - 4.4 Microbial Ingress Risk Comparison
   - 4.5 Negative and Positive Controls

1. INTRODUCTION

Package Integrity Evaluation—Sterile Products ⟨1207⟩ provides guidance in the integrity assurance of product packages. This chapter describes package integrity verification during three product life cycle phases: 1) package development, package processing, and assembly validation; 2) product manufacturing; and 3) commercial product shelf-life stability assessments. Further, this chapter provides information on how to select, develop, and validate leak test methods.

2. PACKAGE INTEGRITY TESTING IN THE PRODUCT LIFE CYCLE

Appropriate packaging for a sterile product can be determined on the basis of a knowledge-gaining effort conducted during the product's life cycle.

2.1 DEVELOPMENT AND VALIDATION

2.1.1 PACKAGE DEVELOPMENT

Package development begins with the preparation of a product–package profile (e.g., user requirements specification), which considers the product end use, stability requirements, and method of manufacture, as well as the anticipated storage, shipment, and distribution environments. This profile also defines the proper product–package quality requirements and the package's maximum allowable leakage limit (see *Package Integrity Evaluation Sterile Products* 〈1207〉, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit*). With this prospectively developed body of information, one can select each package component's materials of construction, choose suitable component sources, and establish critical physical attributes and component dimensional tolerances. Each component material, with its critical dimensional tolerances, directly affects the integrity of the final packaged product. Assurance of package integrity originates from the use of appropriate materials, accurate and optimum closing properties, matching dimensional fit, appropriate multi-component stack heights and tolerances, and consistent control of processes used to assemble the closed package.

The manner in which the package is processed, formed, or assembled is an important consideration in package integrity assurance. A preliminary assessment of package integrity at the end of the development phase under conditions representative of the marketed product manufacturing system is prudent.

These conditions include processes such as sealing operations and component sterilization. Where possible, processes are performed according to established and approved user requirement specifications. Establishing appropriate physical characteristic specifications for container materials, considering lot-to-lot variations, can ensure that the most extreme processing conditions anticipated (e.g., multiple sterilization cycles) do not physically damage materials in a manner that would adversely affect package integrity.

Finally, the robustness of the manufactured product–package system may be evaluated during the development phase by exposing a representative number of product samples to specified storage, shipment, distribution, and final product-use environments. These efforts may include studies that evaluate package integrity at the extremes of the finished product–package profile, not simply at optimal conditions. Given the complexity of some sterile packages, it may be useful for package integrity development studies to incorporate multi-point analyses of test packages manufactured within specified process parameters. Utilizing a common container–closure for which the producer has significant experience and process knowledge can supplement the overall development process and may help reduce the efforts needed. A battery of integrity and seal quality test methods may be employed during product–package development, starting with techniques able to measure the product's inherent package integrity. Inherent package integrity of a viable package system conforms to the product's maximum allowable leakage limit.

Test package quantities for inherent package integrity verification may vary on the basis of: 1) the complexity of the product–package, 2) the specifics of the user specification requirements, and 3) the prior experience of the producer. Test quantity choice is also influenced by the confidence that can be placed on the package integrity test results as well as the level of integrity assurance desired. In some cases inherent package integrity verification may be more readily and economically determined by using empty or placebo-filled container–closure systems, thereby enabling larger sample quantities to be tested by the most sensitive and quantitative leak test methods without the risk of product formulation interference with the test method.

The outputs of the packaging development phase include the final user requirement specifications, which form the basis of production purchasing specifications for package components. Also during the development phase, the final equipment user requirement specifications are developed for package material cleaning, sterilization, and forming; sealing or assembly equipment; and allied materials supply and component feed systems. These user requirement specifications provide purchase specifications for the acquisition of equipment or for the vetting of potential contract manufacturers.

2.1.2 PACKAGE PROCESSING AND ASSEMBLY VALIDATION

Final confirmation of acceptable inherent package integrity is generally part of a larger process validation activity for the overall production process. The scope depends upon the product type and whether the organization has previous experience with the container–closure system.

All processes germane to the sterilization and formation of a package having integrity are to be evaluated against the user requirement specifications established in the package development phase, including likely process extremes. For example, inherent package integrity verification may consider extremes of package assembly variables such as line speed, heat-sealing temperature, screw-cap application torque, and vial-capping forces, as well as resterilization processes, labeling, and secondary and tertiary packaging processes. Validation test requirements and scope should fit the statistical requirements and capabilities of each process, taking into account both package and package-line complexity, as well as prior experience with similar product–packages.

Testing done during technical transfer from the product development site to the manufacturing sites will assist in determining whether the user requirement specification targets and the control ranges established in development require any modification when packages are made on a full manufacturing scale.

Integrity test methods for package processing and assembly validation studies are meant to verify the packages' continued conformance to the product's maximum allowable leakage limit. For some product–packages, the most fitting integrity test

methods for this life cycle phase may have a detection limit greater than the maximum allowable leakage limit. Methods able to reject largely leaking packages such as those caused by defective or out-of-specification components, package damage, and/or package misassembly are appropriate. Seal quality tests suitable for use include those able to monitor package processing and/or assembly consistency.

Successful validation will result in a package that meets its user requirement specifications. The primary objective in package development and subsequent validation is to arrive at a quality product–package prepared using processes that reliably and consistently run within specified operating parameters as defined in the user requirement specifications, yielding critical package defects at a satisfactorily low rate. When performed, in-process and end-product package integrity testing should complement, not replace, thorough package development efforts.

2.2 Product Manufacturing

To ensure the quality of the manufactured product–package, it is critical to specify components of sufficient quality and to select vendors carefully. The factors to consider include:

- Acceptable results of the initial vendor or supplier evaluation
- Appropriate vendor acceptance quality limits and statistical sampling plan(s), or relevant certification
- Incoming component quality verification, including statistical assessment of quality against purchase specifications
- Appropriate procedure(s) for establishing corrective and preventive action when a vendor falls short of quality expectations

Package integrity re-evaluation is considered when changes occur in the product, package design, package materials, or manufacturing/processing conditions. The extent of change control efforts are evaluated on a case-by-case basis, while the level of testing required to support the change is determined based on impact assessment.

The product's package integrity profile is compiled over the course of commercial manufacture. This database of ongoing leak and seal quality test results serves to flag potentially harmful package integrity excursions that may be linked to operative variations in package component design/material and package assembly/processing. Not all packaged-product damage can be readily detected post-assembly; therefore, processing procedure controls and monitors may be relied upon to safeguard against such problems. The proper combination of package leak tests, complementary package seal quality tests, and visual inspection is largely based on package failure results observed in earlier production validation and package development studies.

Testing production lot samples can provide a measure of package integrity confirmation, while entire testing by nondestructive means is able to yield an ongoing assessment of integrity assurance. In some cases regulatory requirement dictates the level of testing performed. For other product–packages, justification for the level of testing is based on statistical process control results generated during the validation phase, and later, on the basis of routine manufacturing product-quality trending analyses.

For example, glass or plastic ampules closed by heat fusion are customarily subjected to 100% nondestructive leak testing. Products sealed under vacuum require appropriate package assembly validation supplemented by testing over time to ensure that the vacuum is retained. Similarly, integrity assurance of packages that require a specific, non-reactive, inert gas headspace is based on appropriate package assembly validation along with testing for rise in reactive gas or water vapor content over time.

2.3 Commercial Product Stability

Container–closure integrity tests have been recommended as alternatives to sterility testing as part of commercial product stability programs. The goal is to ensure package integrity as a function of long-term product storage (*1*). [Note—Package integrity tests do not replace product release sterility tests.] Testing product for sterility is a poor measure of product–package integrity and also will not ensure product–package integrity over the shelf life of the product when performed as part of the stability program. In addition, a package may be in no danger of microbial ingress and yet be unable to maintain the gas headspace content necessary for product quality. Validated package integrity test methods using technologies such as those described in this chapter are more sensitive and reliable than product lot sterility testing for detecting a breach in package integrity that could lead to sterility loss or failure in relevant product physicochemical quality attributes.

Ideally, package integrity tests selected to support marketed-product stability studies are able to verify the absence of the smallest leaks of concern for a given product–package system. In other words, the product's maximum allowable leakage limit falls within the chosen test method leak detection range. However, the test methodology most appropriate for a particular product–package system may be unable to detect the very smallest leaks of concern. In some cases the package contents interfere with the ability of leak test methodologies to detect the smallest leaks. For example, proteinaceous ingredients or even salts may clog leak pathways, inhibiting leak detection by gas flow methods such as vacuum decay or mass extraction. It is prudent to understand the product's potential to interfere with the selected leak test method, both initially after package assembly and over time.

In situations such as those described above, the package integrity test chosen to support stability studies should have a detection limit as close as feasible to the product's maximum allowable leakage limit. An understanding of what the method is capable of evaluating and how this is applicable to microbial integrity assurance is warranted.

General Chapters

For products that demand package headspace content preservation, it is appropriate that the integrity test for stability studies verify the continued presence of specific headspace gases or subatmospheric pressure over time. Satisfactory results verify the absence of leaks that could jeopardize product sterility as well as relevant physicochemical quality attributes. The required duration for monitoring container vacuum or headspace gas content maintenance that equates to microbial barrier assurance and liquid product loss prevention can be predicted on the basis of gas flow kinetics, should a leak pathway exist that is large enough to allow microbial entry and product formulation loss.

Stability test samples intended for package integrity evaluation are kept at labeled storage conditions for the marketed product. Test sample quantities chosen for each testing time point are to be relevant and sufficiently representative of the purpose of stability testing, which is to indicate whether integrity is affected by the stability conditions. Sample quantity selection takes into account all prior development and validation testing.

As noted in reference (*1*), if an integrity test is nondestructive to the product or package, samples that pass package integrity testing may be further used in the stability testing for that specific test period or interval. However, samples should not be tested for package integrity at one time point (e.g., 12 months), then stored for further stability testing at a later time point (e.g., 24 months). While not specifically noted in reference (*1*), it is logical to assume that test samples earmarked for integrity verification over the course of the stability study could be checked for integrity before placement on stability if a nondestructive leak test method is used. This would be akin to the common practice of visually inspecting test samples prior to placing them on stability. In this way, subsequent integrity failures can be attributed to stability storage, rather than to other causes.

3. TEST METHOD SELECTION CRITERIA

No single package leak test or package seal quality test method is applicable to all product–package systems. Test method selection is made on a product–package on a case-by-case basis. Often more than one test method is employed during a given product's life cycle. Package test method selection as a function of product life-cycle phase, along with important integrity considerations, are discussed in *Package Integrity Testing in the Product Life Cycle*. A broad discussion of additional selection criteria for leak test methods follows. Specific leak test method examples are provided for informational purposes but are not intended as recommendations. The attributes and general capabilities of tests can be found in *Package Integrity Leak Test Technologies* ⟨1207.2⟩ and are helpful in the method selection process.

3.1 Package Contents

When selecting a leak test method, the first determining factor is the nature of the package contents. Whether the package contains a liquid or solid formulation, and whether it contains a headspace of inert gas, air, vacuum, or even no headspace at all, will influence leak test method choice. For example, if testing liquid-filled packages by vacuum decay or mass extraction, test vacuum conditions may trigger some formulations to solidify inside leak paths, thereby blocking gas leakage flow and making the test ineffective. Alternatively, electrical conductivity and capacitance tests can be used, but only if the liquid product is more electrically conductive than the package materials.

3.2 Package Design, Materials of Construction, and Mechanics

Packages vary widely in their design, their materials of construction, and the mechanisms whereby package closure is affected. Each of these variables influences the leak test method choice, as the following examples illustrate.

3.2.1 RIGID OR FLEXIBLE PACKAGES

Rigid (nonporous) packages can tolerate the pressure or vacuum-challenge conditions required in several leak tests, including tracer liquid ingress, vacuum decay, mass extraction, and some tracer gas tests. In contrast, some flexible packages can tolerate differential-pressure test conditions only if special tooling is used to restrict package expansion and prevent subsequent seal damage. Package restriction is necessary to ensure consistent differential pressure conditions across the package barrier.

3.2.2 MOVEABLE OR FIXED COMPONENTS

Moveable package components, such as syringe stoppers (plungers or pistons), may require restraint to prevent their dislocation during the differential pressure test conditions required for a majority of leak test procedures (e.g., tracer liquid test, pressure or vacuum decay test, mass extraction test, bubble emission test, and microbial challenge by immersion test).

3.2.3 POLYMERIC MATERIALS

When exposed to test vacuum conditions, package materials such as plastics and some elastomers may outgas volatiles that raise vacuum-decay leak test results as well as mass extraction results, falsely implicating package leakage. Plastic packages that

are highly permeable to tracer gases may not be compatible with helium tracer gas leak detection, as helium permeating through the package could be mistaken for package leakage or may mask small leaks. Special fixtures to limit tracer gas permeation effects and isolate tracer gas exposure to the seal area under test have been used to mitigate such difficulties.

3.2.4 METALLIC MATERIALS

Packages made of foil laminate materials may prove incompatible with electrical conductivity and capacitance leak detection, which works best with relatively nonconductive package materials. However, aluminum caps used to secure vials closed with elastomeric closures pose no hindrance to electrical conductivity and capacitance tests, even for finding leaks located between the closure and the vial finish.

3.2.5 TRANSPARENT OR OPAQUE MATERIALS

Packages made of transparent or translucent materials allow for visual inspection and electromagnetic wave passage. Therefore, transparent or translucent materials can be tested by laser-based gas headspace analysis techniques, as well as tracer-liquid ingress or microbial ingress. Opaque packages are incompatible with testing approaches that require visual inspection of the package contents.

3.3 Closure Type and Mechanics

Refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Closure Type and Mechanics* for a discussion of the closure type categories represented by the various product–package systems. The design of the closure system and its leakage restriction function (i.e., the maximum allowable leakage), plus the types of defects anticipated, influence the integrity test method choice.

3.4 Maximum Allowable Leakage Limit

The maximum allowable leakage limit as a function of the product–package quality requirement is discussed in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit*. The following integrity test method selection considerations are offered in light of that discussion.

3.4.1 STERILITY AND PRODUCT FORMULATION CONTENT MUST BE PRESERVED; GAS HEADSPACE PRESERVATION IS NOT REQUIRED

Integrity tests for this product quality category include those able to verify that the maximum allowable leakage limit that prevents liquid and microbial ingress is not exceeded. Tracer gas tests performed using the vacuum mode and laser-based gas headspace analysis test methods are two examples. Both have been shown to be sensitive enough to quantitatively analyze leakage through the smallest leak paths found to pose the smallest chance of liquid leakage or microbial ingress in rigid packaging. Such tests have also proven useful for defining relationships among package design, component fit, package assembly parameters, and leakage rate, even in the absence of package defects.

Leak testing of product-filled packages during later product-life-cycle phases often requires other tests. Leak test methods available for this phase of the product life cycle are able to be validated to reliably detect defects a few micrometers and larger. Examples of such physicochemical leak tests include vacuum or pressure-decay tests, mass extraction methods, electrical conductivity and capacitance tests, and liquid tracer tests.

3.4.2 STERILITY, PRODUCT FORMULATION CONTENT, AND GAS HEADSPACE CONTENT MUST BE PRESERVED

For this product quality category, leak test options include those that directly check for package headspace pressure and/or content, such as laser-based gas headspace analysis techniques. The detection limit for such methods is a function of the method's ability to accurately measure package headspace content or absolute pressure at the product acceptance limit, given the package headspace volume and the time lapse after package assembly. Such methods have broad application throughout the product life cycle.

3.4.3 STERILITY MUST BE PRESERVED; PRODUCT ACCESS IS REQUIRED

The product quality subcategory relates to the additional integrity requirement for those products that are contained in multiple-dose packages once the product is accessed by the end user. As discussed in *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit, Sterility Must Be Preserved; Product Access is Required*, physicochemical as well as microbiological test methods designed to characterize and verify the unique barrier properties specific for the given container–closure system and its end-use application may be required (*2*).

General Chapters

Elastomeric closures of multiple-dose parenteral products are meant to reseal, limiting microbial ingress and product leakage during and between product access via needle puncture. The test in *Elastomeric Closures for Injection* 〈381〉, *Functionality Tests, Self-Sealing Capacity* is a blue dye liquid tracer test intended to screen closures for their ability to prevent gross liquid leakage post puncture. Caution is advised when solely relying on this test to prove a given closure's ability to reseal. Other test method(s) may need to be designed and implemented that more fully characterize the closure's leak prevention capabilities for the given product–package system and product end-use application.

3.5 Deterministic or Probabilistic Methods

A "deterministic leak test method" is one in which the leakage event is based on phenomena that follow a predictable chain of events, and leakage is measured using physicochemical technologies that are readily controlled and monitored, yielding objective quantitative data. Most deterministic leak test methods rely on the predictable movement of gas that inevitably occurs through an open leak path, given specific differential pressure and/or partial pressure test conditions (e.g., tracer gas, laser-based gas headspace analysis, pressure decay, vacuum decay, and mass extraction). The electrical conductivity and capacitance test is a deterministic leak test approach that relies on the more predictable presence of liquid near a leak path, rather than the less predictable movement of liquid through a leak. Deterministic methods are characterized as being capable of reproducibly detecting leaks at clearly defined and predictable detection limits. Because the majority of deterministic leak test methods described in this chapter require no special test sample preparation, sample preparation error is eliminated.

A "probabilistic leak test method" is stochastic in nature in that it relies on a series of sequential and/or simultaneous events each associated with uncertainties, yielding random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate larger sample sizes and rigorous test condition controls to obtain meaningful results. Typically, sample size and test condition rigor are inversely related to leak size. Therefore probabilistic leak test methods are more challenging to design, develop, validate, and implement, especially when used to find leaks near the upper and lower limits of the test method's detection range. Probabilistic methods include microbial challenge tests, as well as some physicochemical tests. These methods include bubble emission tests, tracer liquid tests (employing either qualitative or quantitative measurement methods), and tracer gas tests by the sniffer probe approach.

A deterministic leak test method having the ability to detect leaks at the product's maximum allowable leakage limit is preferred when establishing the inherent integrity of a container–closure system. Deterministic methods may also be chosen if test sample quantities are limited, when checking for rarely occurring leaks of concern and/or when the potential risk for failing to find leaks of a given size or type is too great. Probabilistic methods are best chosen when the method outcome requirements demand a specific probabilistic testing approach. Probabilistic tests are further discussed in *Method Outcome* and *Quantitative or Qualitative Methods of Analysis* below.

3.6 Physicochemical or Microbiological Methods

"Physicochemical leak test methods" are those that use physical and/or chemical analysis techniques to test for package integrity. Physicochemical methods include both deterministic and probabilistic leak test methods. "Microbiological leak test methods" are probabilistic methods of analysis that use viable microorganisms to evaluate test sample integrity. This chapter includes the microbial challenge test by immersion exposure.

The following section includes additional information on the test method outcome criterion that is related to the microbiological challenge test method.

3.7 Method Outcome

The outcome(s) sought from leak test results will often drive the test method choice. These desired outcomes may include:

- Leak path presence of detection
- Leak path location determination
- Leakage rate measurement
- Liquid egress/ingress potential
- Microbial ingress potential

All leak test methods are meant to identify the leak path presence. Often, leak tests are able to provide additional outcome information. Methods that also provide evidence of leak path location include electrical conductivity and capacitance tests, bubble emission tests, tracer gas sniffer probe tests, and some tracer liquid tests. Methods that provide a measure of whole-package leakage rate include laser-based gas headspace analysis, mass extraction, pressure and vacuum decay methods, and tracer gas tests by the vacuum mode.

Microbial challenge tests provide information on the degree of protection afforded by the product–package against microbial ingress that occurs via active growth or motility through leak pathways and/or by liquid carrier passive transport through leak pathways. Microbiological challenge tests help to clarify the risks to product sterility posed by specific package materials, package designs, or potential package barrier breaches. Sterility risks linked to particular environmental exposure or product use conditions may also demand a microbiological challenge methodology. In summary, all leak test methods at minimum

General Chapters

detect leaks; some methods may provide more information, but no single method alone can yield all four outcomes listed above.

3.8 Quantitative or Qualitative Methods of Analysis

The measure of analysis can play a part in leak test method selection. In other words, does the method yield quantitative data that allow for objective analysis, or are the data strictly qualitative and require more subjective interpretation? Leak test methods that use a "quantitative measure of analysis" include electrical conductivity and capacitance tests, laser-based gas headspace analysis, mass extraction, pressure and vacuum decay, tracer gas tests (especially when testing via the vacuum mode), and tracer liquid tests that use quantitative analysis (e.g., spectrophotometric analysis).

Conversely, a "qualitative measure of analysis" is based on subjective observation of a specific quality, attribute, or characteristic of the test sample, e.g., a visual check for turbidity when evaluating microbial challenge test samples. Bubble emission tests that report visible evidence of continuous bubbling, and tracer liquid tests that rely on visible evidence of dye migration are other examples of subjective and qualitative analysis. Because qualitative measurement results are subject to interpretation, they may be prone to human error. When method considerations permit, leak test methods that yield quantitative measurements are preferred.

3.9 Leak Test Detection Limit

The detection limit of a leak test is the smallest leakage rate or leak size that the method can reliably detect, given the product–package of interest. A large variety of measurement units are used to describe leakage rates and leak sizes when specifying the detection limits (and detection ranges) of leak test methods. This often leads to confusion when comparing the performance claims of various instrument manufacturers or examining test results generated by multiple methods.

To address this, *Table 1* presents the relationship between orifice size (assuming a perfect hole of negligible length) and the rate at which dry air would pass through such a hole when exposed to 1 atmosphere (atm) differential pressure at a specified temperature.

These leakage rates and leak sizes are theoretical approximations and are not definitive.

Table 1 services two purposes. First, it is meant to help the reader better grasp the relationship between theoretical hole diameter and the gas leakage rate. Second, it provides a common measurement scale that can be referred to later in this chapter to more simply state leak detection limits for the various technologies described. For example, test technology X found in published studies to detect leaks as small as about 8 µm would be referred to in ⟨1207.2⟩ as having an approximate leak detection limit of row 4 in *Table 1*.

Leak detection limit should not be the only or perhaps even the primary basis for choosing a test method. Often, the best method for a given application is dictated by other factors. For example, a tracer gas leak test method having an extremely small leakage rate detection limit may be the proper choice for establishing the inherent package integrity of a stoppered glass vial as a function of capping machine parameters during package development. Yet this method would be an inappropriate choice for rapid on-line testing in routine manufacturing. Instead, an electrical conductivity and capacitance test with a larger leak detection limit, able to test product-filled packages at on-line speeds, may be the best option. Refer to *Detection Limit* for a discussion on how to determine the limit of detection.

Table 1. Gaseous Leakage Rate versus Orifice Leak Size[a]

Row	Air Leakage Rate[b] (std·cm^3/s)	Orifice Leak Size[c] (µm)
1	$<1.4 \times 10^{-6}$	<0.1
2	1.4×10^{-6} to 1.4×10^{-4}	0.1 to 1.0
3	$>1.4 \times 10^{-4}$ to 3.6×10^{-3}	>1.0 to 5.0
4	$>3.6 \times 10^{-3}$ to 1.4×10^{-2}	>5.0 to 10.0
5	$>1.4 \times 10^{-2}$ to 0.36	>10.0 to 50.0
6	>0.36	>50.0

[a] This table is not intended for ranking test methods but is offered as an aid for expressing test method leak detection capabilities in this chapter.

[b] Dry air leakage rate measured at 1 atm differential pressure across an orifice leak (i.e., leak inlet pressure of 1 atm versus outlet pressure of approximately 1 Torr) at 25°. The theoretical correlations of orifice sizes to air leakage rates were provided by Lenox Laser, Glen Arm, MD. Leakage rates are approximation ranges.

[c] Nominal diameter orifice sizes assume a leak path of negligible length. Orifice sizes are approximation ranges.

3.10 Leak Test Method Range

Leak test method range is the interval between the smallest and largest leak size (or leakage rate) that can be detected by a given leak test method with a suitable level of accuracy and precision. All leak test methods have an optimum detection range. Therefore, it is possible that additional tests may be needed to catch those leaks of concern that fall outside the chosen leak test method's detection range. For example, a test method able to find the smallest leaks may miss gross leakage such as a missing package component. Vision systems that check for major package defects such as a missing package component or a

General Chapters

package crack may be required. Finally, before using a test method to check for large leaks, the impact of gross defects on leak test instrumentation should be considered; some instruments may malfunction or become damaged upon exposure to a leaking product or damaged packages. Refer to *Range* for additional detection range information.

3.11 Nondestructive or Destructive Methods

The need to preserve the test product–packages may influence the decision to select a nondestructive leak test method, rather than a destructive one. Destructive test methods damage the test sample and/or expose it to potential contaminants; hence, the product is not recoverable. Only nondestructive test methods are appropriate for leak testing product–package units earmarked for commercial or clinical study distribution.

Examples of destructive leak test methods include tracer liquid ingress tests, bubble emission tests, and microbiological challenge tests. Tracer gas and pressure decay leak tests are considered destructive if they require that the package barrier be compromised in order to introduce tracer or pressurizing gas into the assembled package. Examples of nondestructive leak test methods include mass extraction and vacuum decay leak tests, as well as noninvasive gas headspace analysis tests. Electrical conductivity and capacitance leak tests are deemed nondestructive if it can be shown that electrical current exposure causes no harm to the product; in rare instances, exposure has triggered headspace ozone formation, causing product oxidation (*3*).

3.12 Off-Line or On-Line Methods

Off-line leak testing is generally performed on a stratified random sampling of the product lot, away from the manufacturing line. Off-line package evaluation allows for the use of any validated nondestructive or destructive leak test and seal quality test option compatible with the product–package. Off-line testing can accommodate slower test cycle times; for methods in which test time is a performance factor, a slower off-line test is often more sensitive than its on-line counterpart. Off-line tests typically are less costly to perform as they utilize bench-top or smaller scale equipment without the sophisticated product-handling machinery required to support higher speed on-line processes.

On-line leak testing is commonly integrated into a continuous fill and seal product–package manufacturing process. A prerequisite for an on-line leak test method for entire lot testing is that it be nondestructive to the package and its contents. On-line testing can potentially provide greater assurance that all packages have integrity and can yield instant feedback in the event of package misassembly or breakage, enabling real-time line corrections. For some test systems, incorporating large-scale leak detection equipment into a complex high-speed product–package filling and assembly line can be prohibitive. Higher line speeds leading to shorter leak detection test cycle times can limit test method detection capability. In addition, the impact of instrument downtime on the production run as a result of leaking packages or possible equipment malfunction is an important consideration. A separate leak testing line may be set up outside the sterile manufacturing suite to allow for full lot testing without the complications of leak test integration with package filling/sealing operations. A few examples of on-line leak test technologies include electrical conductivity and capacitance, vacuum decay leak, and noninvasive laser-based gas headspace analysis tests.

4. TEST INSTRUMENT QUALIFICATION, METHOD DEVELOPMENT, AND METHOD VALIDATION

Leak test methods are validated in order to demonstrate method effectiveness. Method validation is preceded by instrument/equipment qualification, followed by test method development. The following discussion specific to leak test methods is intended to supplement the guidance for analytical instrument qualification presented in *Analytical Instrument Qualification* ⟨1058⟩, plus the guidance for method validation provided in *Validation of Compendial Procedures* ⟨1225⟩.

4.1 Instrumentation and Equipment Qualification

The qualification of instruments or equipment to be used for leak testing includes: 1) evaluation of instrument/equipment functionality, and 2) determination of test system detection capabilities using appropriate calibration tools or reference standards to simulate with-leak test conditions.

4.2 Method Development and Validation

After successful instrument/equipment qualification, leak test method parameters are developed and optimized to ensure a leak test method is able to meet all relevant leak detection performance criteria specific for the test product–package system. The following properties of a valid test method are defined as they relate to package integrity tests.

4.2.1 ACCURACY

"Accuracy" is a measure of the method's ability to correctly differentiate packages that leak above the claimed detection limit from those that leak below this limit (i.e., do not leak). Accuracy provides a measure of false positive and false negative

occurrence. Alternatively, for those methods that deliver an outcome that is a direct quantitative measure of gas leakage rate (or of gas concentration or gas pressure), accuracy is a measure of the method's ability to produce an outcome comparable to a true standard. For example, helium mass spectrometry provides a direct measure of helium leakage rate. Accuracy is the closeness of the instrument reading to the certified leakage rate of a nationally recognized traceable standard.

4.2.2 PRECISION

"Precision" is the ability of the method to yield reliable, repeatable data. Precision includes repeatability (e.g., repeat testing of a homogeneous test sample population), ruggedness (within laboratory tests performed, for example, by multiple operators on multiple days, using multiple instruments; also known as intermediate precision), and reproducibility (among laboratories tests). The level of precision to which a leak test method is validated is often a function of resource availability (e.g., one instrument versus multiple instruments) and intended test method application (e.g., use of the method at one test site only versus across multiple test sites).

4.2.3 SPECIFICITY

"Specificity" is the ability of the method to accurately differentiate between leaking and nonleaking packages, despite interfering factors that may cause false detection. For example, when employing tracer gas leak detection using helium mass spectrometry (vacuum mode), excessive helium permeation through the package wall may mask small package leaks or may be falsely interpreted as leakage in no-defect packages.

4.2.4 DETECTION LIMIT

Refer to *Leak Test Detection Limit* for an introduction to this topic. The detection limit of a leak test is specific for a given testing approach when performed using a specific instrument make/model in evaluating a given product–package system. Utilizing the principles described in ⟨1225⟩, detection limit is demonstrated by challenging packages with and without known defects by the leak test method for multiple test days by multiple operators. The intended application of the method will dictate the level of precision required (i.e., whether to incorporate multiple operators/instruments/laboratories, etc.).

A common challenge with small leak detection is the potential for interfering factors to be misconstrued as leakage presence. For example, vacuum decay leak tests measure the rise in pressure inside an evacuated chamber containing the test package. Package leakage causes chamber pressure to rise, but package material volatiles, test system moisture, and package expansion may also do the same. Gas permeating through a package wall detected by a tracer gas test may be mistaken for leakage. Bubbles emitted during a bubble test may actually be the result of package surface outgassing, volatilization of dissolved gases in the immersion fluid, or the release of trapped air between package components.

The absence of a leak detection signal may also be misinterpreted as the absence of leaks. For example, tracer liquid tests may fail to reliably detect small leaks due to any one of a number of factors including air locks, product, or debris in the leak path; liquid surface tension; leak path geometry; or insufficient differential pressure test conditions. The same is true for microbial ingress tests that are further subject to the inherent variability of living microorganisms.

In short, false negative results that miss leaks and false positive results that incorrectly suggest leak presence are possible with any leak test method. Therefore, test method detection limit is determined by comparing readings of intentionally defective packages to nondefective ones. Leak detection limit determination test units consist of a randomly ordered population mix of negative and positive control units (refer to *Negative and Positive Controls*). Nondestructive test methods can employ the same set of units for multiple test exposures, while destructive methods will require a new set for each test series. Control subset unit quantities are chosen based on several factors: 1) the deterministic or probabilistic nature of the outcome, 2) the inherent package-to-package variability that may influence test results, and 3) the statistical confidence level required by the test acceptance criterion. The positive control subset includes units with defects sized to the anticipated detection limit, in addition to units with leaks bracketing this size limit. If the detection range is to be established, controls having large defects are included. Positive controls representing a wide defect size range are especially important for probabilistic methods in order to clearly understand leak detection likelihood as a function of leak size.

Because of the many product–packages and leak-testing options available, the resultant leak detection limit is more meaningfully stated when the negative and positive control subsets used, the test precision level, and the test results are summarized. The following is an example of expressing a test method's limit of detection:

"The detection limit for method X was determined to be 5 ± 2 µm. Validation studies found defects of this nominal size were detected 95% of the time; all larger defects were detected 100% of the time. Studies included three replicate test series performed on multiple days by multiple operators in a single laboratory using one instrument. Detection limit was determined using product-filled packages. Test units in each series included a negative control subset of 300 units (each without defect) and a positive control subset of 90 units (each having a laser-drilled defect ranging in nominal size from 1.5 ± 0.6 µm to 15 ± 3 µm). Each defect was independently size-certified by comparing the dry air leakage rate at 1 atm differential pressure (leak inlet pressure of 1 atm versus outlet pressure of approximately 1 Torr) at 25° to that of standard orifice leaks."

[NOTE—This is one example of how test method detection limit could be expressed and is not to be considered compulsory or restrictive either in content or level of detail.]

Leak test technologies exist that are able to detect leaks even smaller than can be artificially created in a positive control test set. Two examples follow. In both cases, a limited number of positive controls can serve to verify that the instrument set-up is able to detect leaks of specific type and in specific package locations but not to determine leak size detection limit capability.

- Tracer gas tests by helium mass spectrometry performed in the vacuum mode can detect leaks as small as about 10^{-11} mbar · L/s, which is about 5 logarithmic leakage units smaller than leakage through a hole 0.2 µm in size. For such methods, leakage rate detection ability can only be verified by using nationally recognized gas leakage rate standards to introduce leaks into the test system. Because gas permeation can be mistaken for leaks, it is important to experimentally establish the relationship between tracer gas leakage rate and permeation rate as a function of test cycle time for a given package system.
- Laser-based gas headspace analysis is another approach that may be able to identify the presence of leaks smaller than can be artificially created. For such methods, the limit of detection can be mathematically predicted on the basis of gas flow kinetics and is a function of the time lapse between analyses, and the smallest gas content or pressure change that can be reliably detected by the instrument for the given package system.

4.2.5 QUANTITATION LIMIT

"Quantitation limit" is that lowest leakage rate or leak size that can be determined with acceptable accuracy and precision under the stated experimental conditions. Laser-based gas headspace analysis is a method that may be evaluated for quantitation limit. For most leak test methods, detection limit is more meaningful.

4.2.6 LINEARITY

"Linearity" is the ability of the method to elicit test results that are mathematically proportional to leak path size or leakage rate. Deterministic leak test methods that exhibit linearity include laser-based gas headspace analysis and tracer gas analysis (vacuum mode). Other methods such as vacuum decay, pressure decay, and mass extraction also produce results that correlate to leak size or leakage rate. However, test findings are generally intended to identify leak presence and perhaps to understand relative leak size; they are not typically relied upon for leak size or leakage rate quantitation. Electrical conductivity and capacitance tests and all referenced probabilistic methods are not validated for linearity.

4.2.7 RANGE

Leak detection range is defined in *Leak Test Method Range*. Leak test method range is explored in test method development to better understand leak test method detection limitations. For methods being relied upon to detect leaks within a specified size range, detection at the upper range limit may be confirmed in validation. Range is evaluated by using sets of negative controls and appropriately sized larger-defect positive controls. The large-defect positive control subset may include defects of various types likely to occur for the given product-package system (refer to *Type Defects*).

4.2.8 ROBUSTNESS

"Robustness" is the method's ability to accurately identify leaking versus nonleaking packages despite small but deliberate variations in procedural parameters, providing an indication of the method's suitability during normal usage. One way to evaluate robustness is to perform the test using test parameters bracketing optimal or normal test specifications. Parameters to be varied are those having the greatest impact on test results; variation should reflect instrument performance accuracy. For example, the robustness of a vacuum decay leak test with a test cycle time of 30 s (accurate to within 0.5 s) might be demonstrated during method development by verifying method performance at set test cycle times of 29.5 and 30.5 s.

4.3 System Suitability

One outcome of test method development and validation is the establishment of a system suitability test(s), also called a performance verification test(s). "System suitability" is a manner of ensuring that the leak test method, including all factors that may be subject to variability that may impact test results (e.g., instrumentation, analysts, test sample preparation, and the test environment), is adequately controlled and maintained in such fashion that the method is rugged and robust. System suitability is important for all leak test methods. Extra care may be required for probabilistic methods more prone to variability.

For example, a highly instrumental method such as a mass extraction test may be checked for system suitability by leak testing a master package unit both with and without the added challenge of external air introduced to the test system via a calibrated leak adjusted to the method's limit of detection. If the method requires preliminary test package preparation (e.g., a drying step), then it would be appropriate to demonstrate that an intact package prepared in the prescribed manner elicits the

anticipated mass flow rate response. System suitability can be performed at the beginning and end of each testing sequence for added method assurance.

System suitability for a test more probabilistic in nature takes into account multiple test method variables and may require greater numbers of challenge samples (i.e., positive and negative controls) for adequate method assurance. For example, tracer gas tests via a sniffer probe may require a routine demonstration that the operator is able to successfully differentiate packages without defect from those with leaks (ranging in size from smallest to largest, located at various package positions) in a blinded challenge study. Alternatively, the operator could be challenged by randomly introducing defective samples (unknown to the inspector) among the test sample population during routine test procedures.

4.4 Microbial Ingress Risk Comparison

Appropriate leak test method validation practices mirror compendial analytical test validation guidelines. In the past, validation of physicochemical leak test methods used for pharmaceutical sterile product packages also routinely included a direct or indirect comparison of physicochemical leak test results to microbiological challenge data. While an understanding of the relationship between physicochemical leak test method capabilities and microbial ingress risk may be important, an experimental comparison may not be needed or useful in all cases. [NOTE—The reader may refer to *Package Integrity Evaluation—Sterile Products* ⟨1207⟩, *Product–Package Quality Requirements and the Maximum Allowable Leakage Limit* for a discussion of maximum allowable leakage limit and inherent package integrity.]

The following situations illustrate when a comparison study of the microbial ingress or liquid leakage risk to physicochemical leak test method capability relationship is likely not needed. [NOTE—This is not intended to be a complete or exhaustive listing of all situations for which comparison studies would not be required or useful but is provided for illustration only.]

- If the validated physicochemical leak test method has a proven detection limit at or below the product-package maximum allowable leakage limit.
- If the validated physicochemical leak test method is not being used to verify the absence of all leaks of concern. Instead, the method is being used to find leaks notably larger than the maximum allowable leakage limit. For example, a rapid online test shown to reliably detect leaks of 25–150 μm in nominal diameter is being used to reject damaged or misassembled product-filled packages within the method's leak detection range.

The following situations illustrate when an experimental indirect or direct comparison of the microbial ingress risk (or liquid leakage risk) to physicochemical leak test method capability relationship may be useful. [NOTE—This is not intended to be a complete or exhaustive listing of all situations for which comparison studies could prove beneficial but is provided for illustration only. These examples are not compulsory but are provided for instruction only.]

- If the validated physicochemical leak test method is being relied upon to measure or confirm the inherent package integrity of a product–package system, but the method's limit of detection is notably greater than the maximum allowable leakage limit. For example, a package has a maximum allowable leakage limit of less than 6×10^{-6} mbar · L/s as measured by helium mass spectrometry (equivalent to holes less than approximately 0.2 ± 0.1 μm in nominal diameter). This limit was chosen based on published literature references. But the leak test method of choice for testing product-filled packages placed on stability is able to detect leaks equivalent to holes 3 μm in nominal diameter and larger. A study correlating microbial ingress or liquid leakage risk to defect type/size can provide a measure of the likelihood of microbial ingress (and/or liquid leakage) at the leak test method detection limit, and thus can provide an understanding of ability of the stability leak test to identify leaks of concern.
- If the validated physicochemical leak test method is being used to measure or verify the inherent package integrity of a product–package system, but the maximum allowable leakage limit is either lacking or not well defined. For example, a unique package is being used for which the maximum allowable leakage limit that will ensure absence of product loss or microbial ingress has not been determined. A study comparing the risk of microbial ingress or liquid leakage to leak type/size, and in turn to the likelihood of detection by the physicochemical leak test method may be useful.

When performing microbial ingress risk assessment studies it is important to keep in mind the probabilistic nature of microbial ingress. To achieve the most meaningful data, large population sets of negative and positive controls should be used. Test protocols should be thorough and well designed, taking into consideration the multiple factors and variables that can influence results. As suggested above, liquid leakage risk assessment studies may substitute for microbial ingress risk studies assuming the risk of liquid leakage is equivalent to or greater than that of microbial ingress, with appropriate justification.

4.5 Negative and Positive Controls

"Negative controls" are packages with no known leak, and "positive controls" are packages with intentional or known leaks. Negative and positive controls are designed and assembled for use in method development and validation with consideration given to container–closure design, materials of construction, characteristics of anticipated package leaks, and impact of product contents on test results. Negative and positive controls should represent packages assembled in a typical manner as the product being tested using normally processed components, the exception being the intentionally created leak in each unit of the positive control subset. Some leak test methods may necessitate positive controls that simulate test product headspace and formulation contents as well. System suitability checks for some test methods employ negative and positive controls (refer to *System Suitability*).

Test blanks should not be confused with negative controls. For example, liquid tracer leak detection by spectrophotometric analysis may require a blank solution without the liquid tracer element to confirm instrument baseline performance.

A "master" is a type of negative control test unit. It is a package prototype, model, or facsimile made to simulate the test package in shape and design. Masters may be made of solid material such as plastic or metal, or they may be simply a designated container–closure unit. Masters are no-leaking mock packages often used in system suitability verification tests for leak tests to verify instrument performance, such as for vacuum decay or mass extraction testing.

4.5.1 DEFECT CREATION METHODS

The positive control set typically represents a range of package defect sizes and types. Numerous approaches have been used to create package defects. When creating positive controls, a fundamental awareness of leakage dynamics as a function of different defect types and materials of construction is important.

Placing a so-called "hole" or break in the package wall is one positive control creation approach. In this case the defect materials of construction are identical to the package itself, thus potential test method interference due to product exposure to the package material can be readily identified. An example of such interference is the clogging of small leak paths by product formulation observed during vacuum decay, mass extraction, or tracer gas (vacuum mode) tests.

Laser drilling is often used to create package defects commonly referred to as holes. However, these so-called holes are not pristine orifices but are non-cylindrical and asymmetric and may consist of a tortuous matrix of micro-cracks. While laser-drilled defects are not dimensionally ideal, they offer the advantages of closely simulating actual package defects (e.g., cracks) and do not require the introduction of foreign materials, such as wires, tubes, or epoxies, that may influence leakage dynamics. Laser-drilled defects are often sized for nominal diameter by comparing dry air leakage rate through the defect at specified conditions of pressure and temperature to flow rates through standard orifice leaks in thin metal plates. Currently, laser-drilled defects in rigid glass or plastic components can be made as small as about 2–3 µm in nominal diameter and to about 5–10 µm in nominal diameter in flexible thicker wall package materials. Smaller diameter defects tend to clog from handling, environmental debris, or flexible package wall deflection.

Glass micropipettes can be used to simulate single-orifice defects as small as about 0.1 µm in diameter. The tip diameter can be nominally sized using air flow measurements. When creating positive controls, micropipettes are inserted through a break in the package wall, then an appropriate sealant is applied to the insertion site. Challenges to micropipette use include ensuring a complete seal between the micropipette perimeter and the package wall and avoiding micropipette tip damage.

In addition, air trapped in the pipette tube can interfere with leak test methods that depend on fluid flow through the leak path, such as tracer liquid tests and microbial challenge ingress tests.

Microtubes (also called microcapillaries) inserted through the package wall and fixed in place with sealant are another means of creating positive control defects. Microtubes can be made of a variety of materials, can be cut to any length and can be as narrow as 2 µm in cross-sectional diameter. Microtubes are often employed as a substitute for a smaller-bore, shorter-length leak path when performing leak tests that rely on gas flow measurements. However, caution is advised before choosing lengthy, larger-bore microtubes to simulate an orifice leak of smaller diameter for leak tests that rely on the passage of liquids or microorganisms. Fluid dynamic theory correlating fluid flow through capillary tubes to pass through smaller bore holes is based on the unimpeded passage of ideal liquids through capillaries at equilibrium pressure conditions. Liquid product formulation and aqueous media flow into and through a microtube is complicated by numerous factors including liquid surface tension, liquid viscosity, surface contact angle, airlocks, particulate blockage, and tube-wall and tube-end finishes. Microbial ingress through microtube defects relies more on the presence of liquid in the tube than on the physical barrier to passage or grow-through afforded by the tube diameter (*4*). Microtubes are a logical choice when creating defects representing channel defects. In this case microtube length should mimic as closely as possible the actual package barrier thickness (package wall or seal width). Microtube use challenges include effecting smooth, perpendicular cuts of microtube ends and adequately sealing microtubes into the test sample wall.

Other commonly used defect creation methods include inserting a needle through the package wall; placing a wire, microfilament, or film between sealing surfaces; and adhering a holed, thin metal plate onto package surfaces. It is important to note that defects made by using an object foreign to the package (e.g., needle, film, wire plate) may display gas, liquid, or microbial leakage dynamics markedly different from that of actual defects (*5*). Positive controls made by such means are easy and inexpensive approaches for creating larger size defects useful for test method feasibility studies and for exploring a test method's detection range upper limit.

4.5.2 TYPE DEFECTS

"Type defects" are positive controls representing realistic package flaws. Type defects are important to include in method validation studies to explore a leak test method's practical application in detecting realistic package failures and flaws. A few types of defect examples are listed below:

- Heat sealed bag: 1) weak seal, 2) wrinkled seal, 3) seal gap, 4) seal channel, 5) product entrapment in seal
- Stoppered vial package: 1) vial finish channel defect, 2) loosely capped stopper, 3) product trapped between stopper and vial
- Prefilled syringe: 1) needle shield punctured by staked needle, 2) defective plunger

General Chapters

• Ophthalmic dropper bottle: 1) loose cap, 2) missing or poorly inserted dropper tip, 3) defective tip or cap

Assigning precise sizes to type defects is generally not meaningful or feasible because of their inherent irregularity and complexity. Type defects are defined in more qualitative, descriptive terms such as those listed above. Because no leak test method can find all possible defects in a given container–closure system, information collected from type defect tests can be used to identify alternative approaches to detecting or limiting the occurrence of critical imperfections not readily found by the chosen leak test method.

REFERENCES

1. Department of Health and Human Services. Container and closure system integrity testing in lieu of sterility testing as a component of the stability protocol for sterile products. Guidance for industry. Rockville, MD: U.S. Food and Drug Administration, Center for Biologics Evaluation and Research (CBER), Center for Drug Evaluation and Research (CDER), Center for Devices and Radiological Health (CDRH), Center for Veterinary Medicine (CVM); 2008.
2. Bagel S, Wiedemann B. Extension of in-use stability of preservative-free nasalia. *Eur J Pharm Biopharm.* 2004;57(2):353–358.
3. Bigwarfe P Jr, Domin-Turza A, Hwang E, Leidner S, McGinley C, Olson K, Nachtigall M. Presentation. Annual Meeting of the American Association of Pharmaceutical Scientists 2008. Elucidation of an unusual mechanism of drug product degradation caused by a leak detection instrument. Lake Forest, IL: Hospira; 2008.
4. Keller S, Marcy J, Blakistone B, Hackney C, Carter H, Lacy G. Application of fluid and statistical modeling to establish the leak size critical to package sterility. *J Appl Pack Res.* 2006;1:11–21.
5. Morrical BD, Goverde M, Grausse J, Gerwig T, Vorgrimler L, Morgen R, Büttiker J-P. Leak testing in parenteral packaging: establishment of direct correlation between helium leak rate measurements and microbial ingress for two different leak types. *PDA J Pharm Sci Technol.* 2007;61(4):226–236.

〈1207.2〉 PACKAGE INTEGRITY LEAK TEST TECHNOLOGIES

1. INTRODUCTION
2. DETERMINISTIC LEAK TEST TECHNOLOGIES
   - 2.1 Electrical Conductivity and Capacitance (High-Voltage Leak Detection)
   - 2.2 Laser-Based Gas Headspace Analysis
   - 2.3 Mass Extraction
   - 2.4 Pressure Decay
   - 2.5 Tracer Gas Detection, Vacuum Mode
   - 2.6 Vacuum Decay
3. PROBABILISTIC LEAK TEST TECHNOLOGIES
   - 3.1 Bubble Emission
   - 3.2 Microbial Challenge, Immersion Exposure
   - 3.3 Tracer Gas Detection, Sniffer Mode
   - 3.4 Tracer Liquid

1. INTRODUCTION

The purpose of this chapter is to provide information guiding the selection and proper use of leak test technologies (also called methodologies, approaches, or methods). The leak test technologies described in this chapter were selected on the basis of relevant research study data published in peer-reviewed journals and/or precision and bias study data generated in support of recognized test method standards. When referencing standard test methods (e.g., ASTM), the reader is advised to refer to the most recent versions. In some cases, the scope of referenced standard test methods does not include the package types of the scope in *Package Integrity Evaluation—Sterile Products* 〈1207〉. In all cases, methods and literature studies are cited to provide benchmark information useful for pharmaceutical package leak test method development and validation.

The technologies described in this chapter are not prescriptive methods but represent testing concepts that may be applied when leak testing sterile product–packages. Test technologies vary in terms of their potential detection limits, reliability, and applications; therefore, none are universally appropriate for leak testing all product–packages. This chapter provides information to allow a thorough comparison of testing approaches so that the most appropriate technology for a given situation can be identified.

After a methodology has been selected for use, the test equipment operation and performance is qualified. Test method parameters are optimized during method development and confirmed during validation. Thus, a final leak test method is specific to a particular container–closure or product–package system.

The leak test methods included are divided into two categories: deterministic and probabilistic. Deterministic leak test methods (*Table 1*) are preferred over probabilistic methods when other key method selection criteria permit. Probabilistic leak test methods (*Table 2*) are best used when the product–package system proves incompatible with deterministic methods, or when method outcome requirements demand a particular probabilistic testing approach.

In this chapter's *Table 1* and *Table 2*, the "leak size detection limit" provided for each methodology refers to leakage rates/leak sizes listed by row in *Package Integrity Testing in the Product Life Cycle—Test Method Selection and Validation* 〈1207.1〉, *Table 1*. The reported leak detection limits were chosen on the basis of literature sources, as well as commonly accepted experience. This information is intended to aid in the selection of the test technology but should not be used as a definitive statement of test method performance for any specific leak test method applied to any given product–package system. Instead, leak detection limit and range should be established during leak test method development and validation for the respective product–package or container–closure system. For instance, an approach cited as capable of detecting row 6 leaks may be validated by the user to detect leaks as small as those in row 3. Conversely, a method described as capable of detecting leaks in row 3 may be determined by the user to detect leaks only as small as row 5.

This battery of testing technologies and the information provided are intended to aid, not limit, the selection, development, validation, and use of leak test methods. Unlisted methodologies shown by the user to meet the qualification and validation requirements for a satisfactory leak test may be used. In addition, listed technologies may demonstrate expanded testing capabilities beyond those currently identified.